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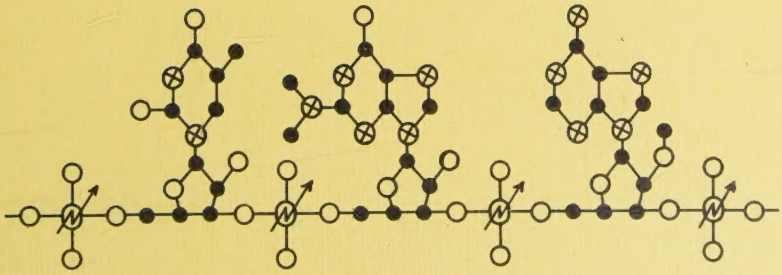
STUDIES ON AMINOACYLATION AND METHYLATION OF TRANSFER RIBONUCLEIC ACID

BY

INGVAR SVENSSON



STUDIES ON
AMINOACYLATION AND METHYLATION
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PREFACE

This work has been carried out at the Institute of Biochemistry, Uppsala, and I am greatly indebted to Professor Arne Tiselius for his kind interest and support. I am also very grateful to Professor Hans G. Boman for introducing me into the field of molecular biology and for many stimulating discussions throughout the progress of this investigation. My thanks are due also to my colleagues, Drs. Glenn Björk, Annika Johansson, Karl-Erik Johansson and Per Lundahl, for their devoted cooperation. I also wish to thank Dr. Walter Björk for his encourageing interest in my work, and Drs. Kerstin Kjellin-Stråby, Leif Isaksson, John Phillips and Stellan Hjertén for many helpful discussions. The skilful laboratory assistance by Miss Birgit Krey is gratefully acknowledged. I am also indebted to members of the scientific and technical staff of the institute for helpful cooperations.

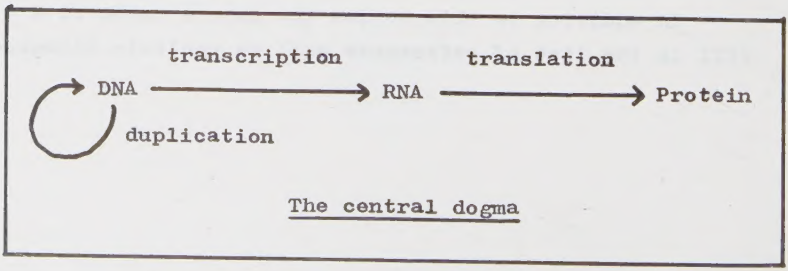
The work has been supported by grants from the Swedish Cancer Society.

In addition to this review the papers numbered I - VIII in the list of references will be publicly discussed.

The last ten years have witnessed the inspiring and sometimes dramatic rise and prosperity of a new discipline of science, molecular biology. Endowed with a pioneering spirit, the molecular biologists have set out to unravel the mysteries of life by an audacious approach to the function of organized cellular systems. One of the most profitable outcomes of this "new way of thinking in biology" is our knowledge of protein and nucleic acid biosynthesis.

Much of the cellular protein constitutes enzymes, which carry out a manyfold of metabolic and regulatory functions of the utmost importance to life. Other proteins are integrating parts of cellular structures, regulatory systems and defence mechanisms. The specific properties and the catalytic action of a protein depend on its three-dimensional structure, which is univocally determined by the sequence of amino acids.

The genetic information for the correct ordering of amino acids in the assembly of polypeptide chains resides in the nucleotide sequence in the deoxyribonucleic acid (DNA) of the chromosomes. According to the central dogma, this information is first transcribed into the sequence of nucleotides in a class of intermediary templates, messenger ribonucleic acid (mRNA), which are then translated by a ribosomal apparatus into the order of amino acids in proteins.



The central dogma

In the process of translation, a class of small RNA molecules, transfer ribonucleic acid (tRNA), plays an important role. Their function is to pick up the right amino acids in the cytoplasm and transfer them to the right positions in the growing polypeptide chains. The tRNA with its load of amino acid diffuses to the ribosome, where it recognizes, by means of a base-pairing mechanism, certain codons in mRNA which correspond to the particular amino acid only.

Aminoacylation of tRNA

The reactions leading to attachment of an amino acid to tRNA are depicted below. The amino acid is first energetically activated by coupling with ATP to form an aminoacyl adenylate, which is firmly bound to the enzyme. The aminoacyl moiety of this complex is then esterified to the 3'-hydroxyl group of the terminal adenylic acid of tRNA:

$$aa_i + ATP + Enzyme_i \rightleftharpoons Enzyme_i\text{-}AMP\text{-}aa_i + PP \qquad (1)$$

$$Enzyme_i\text{-}AMP\text{-}aa_i + tRNA^{aa_i} \rightleftharpoons aa_i\text{-}tRNA^{aa_i} + AMP + Enzyme_i \qquad (2)$$

The enzyme catalyzing these reactions, aminoacyl-tRNA synthetase, has a high degree of specificity in its recognition of both the amino acid and the tRNA molecule to carry it. For each of the twenty amino acids making up the proteins there are at least one enzyme of this kind and one or more tRNA chains (1-5).

The author has studied the methionyl-tRNA synthetase from two different organisms, the yeast Saccharomyces cerevisiae and the bacterium Escherichia coli. Some peculiarities met with in early work (6) prompted a thorough investigation of the cation requirements of these enzymes. It is shown in paper I that the yeast enzyme is activated by a variety of divalent metal ions, but the optimal ion concentration may

differ greatly with the kind of metal ion and also with the kind of tRNA used as methionine acceptor. The rate of methionyl-tRNA formation is stimulated by the presence of some monovalent cations (NH_4^+ and K^+), although these ions have no effect on the rate of methionine activation (reaction 1). The methionyl-tRNA synthetase from E. coli has a similar behaviour, the only exception being that K^+ does not stimulate the rate of methionyl-tRNA formation (paper II).

The maximum yield of aminoacyl-tRNA synthesized is usually a function of the amount of tRNA only. In the heterologous reaction between yeast methionyl-tRNA synthetase and E. coli tRNA, however, there is a clear dependence on both enzyme and NH_4^+ concentration for maximum methionyl-tRNA formation. A characterization of this phenomenon is given in paper III.

Methylation of tRNA

The central dogma states that the genetic information contained in DNA is transcribed, by base-pairing, into the nucleotide sequence of RNA. This mechanism seems to be the same for both mRNA, tRNA and ribosomal ribonucleic acid (rRNA). The methylated purines and pyrimidines present in some of the nucleic acids do not fit into the postulated scheme, however, and the situation remained paradoxical until it was discovered by Fleissner and Borek (7), and, independently, by the present author in collaboration with Dr. Boman that there exists a class of enzymes capable of methylating specificly some of the bases on the preformed tRNA polymer (paper IV). It soon became apparent that there existed enzymes for methylation, on the polynucleotide level, of rRNA and DNA as well (8,9).

Enzymatic mathylation of nucleic acids rapidly became a hot field (10-14), and after the primary characterization of the reaction, much effort was directed towards the problems concerning specificity of the enzymes. Both with crude

extracts (15) and with purified tRNA methylases from E. coli (16) and yeast (papers VI and VII) it has been demonstrated that these enzymes are species specific. The known structures for some tRNA chains indicate that the enzymes are highly specific for the sites of methylation on the tRNA polymer. Much work is still needed to find out, with purified enzymes and tRNA chains, the structural features on tRNA which determine the sites for methyl group attachment.

In both tRNA and rRNA a second class of methylated constituents has been detected, namely 2'-O-methylribonucleosides. The enzymatic synthesis of these compounds on the polynucleotide level, in analogy with the synthesis of the methylated bases, has recently been demonstrated with rRNA contained in ribosomal precursors (17) and with tRNA in a heterologous system (paper VIII).

The biological function of the methyl groups in nucleic acids has been the centre of much speculation. Although many experiments have been designed to elucidate the significance of tRNA methylatinn, no conclusive results have been reported to date. A recent paper discusses this problem, and evidence is presented that submethylated tRNA, specific for phenylalanine, is less reactive in the aminoacylation reaction than normal tRNA (18).

It has been found that some tumour cells have an abnormal degree of nucleic acid methylation (13). The possibility that there is a causal correlation between the genesis of tumours and the aberrant methylation is challenging and deserves further attention. It is also of interest that changes in the methylation pattern of tRNA show up after phage infection of bacteria and in insects during metamorphosis (13). Also, methylation of DNA seems to be at least in part related to the phenomenon of phage restriction in bacteria (19).

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STUDIES ON MICROBIAL RNA.

Fractionation of tRNA methylases from Saccharomyces cerevisiae.

by

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INTRODUCTION

Transfer RNA from all organisms examined so far contains a number of methylated bases in addition to the four ordinary bases. In yeast tRNA, as many as 12 different methylated bases have been detected (Hall, 1965; Littlefield and Dunn, 1958; Dunn, 1963). The occurrence and distribution of these minor components in tRNA seems to differ not only between species but also between strains of the same species (Phillips and Kjellin-Stråby, 1967).

It is now well established that the methylated bases are synthesized at the macromolecular level by a set of specific enzymes and with S-adenosylmethionine as methyl donor (Borek and Srinivasan, 1966; Srinivasan and Borek, 1966; Venkstern, 1967). The study of these enzymes was simplified by the finding that a methionine-requiring auxotroph of Escherichia coli K12 with relaxed control of RNA synthesis, when deprived of methionine, produces methyl-deficient tRNA (Mandel and Borek, 1961), which can serve as substrate for methylases from a variety of organisms (Fleissner and Borek, 1962; Srinivasan and Borek, 1963). The tRNA methylases are, however, species specific and can in general find new sites for methylation in heterologous tRNA, which already has its normal supply of methyl groups.

Most of the work on tRNA methylases has been done with crude extracts containing all the various enzymes. Only one successful attempt at fractionation has been reported to date. This work on E. coli tRNA methylases revealed the existence of at least six different enzymes (Hurwitz et al., 1964). All efforts to fractionate the tRNA methylases from rat liver have hitherto failed because of instability of the enzymes (Rodeh et al., 1967). In this paper we describe the fractionation of tRNA methylases from yeast.

Abbreviations: 1MA, 1-methyladenine; 2MA, 2-methyladenine; 6MA, N^6-methyladenine; 6DMA, N^6-dimethyladenine; 1MG, 1-methylguanine; 2MG, N^2-methylguanine; 2DMG, N^2-dimethylguanine; 7MG, 7-methylguanine; 1MU, 1-methyluracil; 5MU, 5-methyluracil(thymine); 1MC, 1-methylcytosine; 5MC, 5-methylcytosine; MUp, methylated uridylic acid; MCp, methylated cytidylic acid; MU, methylated uracil; MC, methylated cytosine; 1MH, 1-methylhypoxanthine; r as suffix denotes mucleoside; GSH, glutathione; SAM, S-adenosylmethionine; 2-ME, 2-mercaptoethanol; tRNA (5MU) methylase, a tRNA methylase producing 5MU.

MATERIALS AND METHODS.

Chemicals.

Sigma Trizma base reagent grade Tris was used for preparation of Tris-HCl buffers. GSH, ribonuclease-A from bovine pancreas, type IIA, deoxyribonuclease 1 from beef pancreas and alkaline phosphatase from E. coli, type III, were purchased from Sigma Chemical Co. DL-Methionine was obtained from AB KEBO, Stockholm, Sweden. Adenine was from Fluka AG, and uracil from The British Drug Houses Ltd, Poole, England.

(Methyl-^{14}C)S-adenosyl-L-methionine (50.5 mC/mmole) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Markers for characterization of the products formed were generous gifts from Dr. R.K. Robins, University of

Utah, Salt Lake City, Utah 841112 (2MGr), Dr. G.B. Elion, Burroughs Wellcome Co, New York (2MG and 2DMG), Dr. Leif Isaksson, University of Umeå, Sweden (1MGr and 7MGr) and Dr. P.L. Bergquist, Yale University, U.S.A. (1MU, 5MU, 1MC, 5MC). 6MA, 1MA and 7MG were obtained from Sigma Chemical Co, St. Louis, Mo., U.S.A. and 2MA was bought from Fluka AG Chemische Fabrik, Switzerland.

Strains and growth conditions.

Saccharomyces cerevisiae, strain D84 (Kjellin-Stråby and Boman, 1965), was grown in medium Y (Svensson et al., 1963) supplemented with 0.2 % yeast extract (Difco), 0.1 mg/ml of uracil, 0.1 mg/ml of DL-methionine and 2 % glucose. In this medium the generation time was about 2 hr at 30°. The cells were harvested in the exponential growth phase, immediately washed with distilled water and stored frozen at -20°.

Saccharomyces cerevisiae, strain D38 (Phillips and Kjellin-Stråby, 1967), is a mutant lacking the base 2DMG in tRNA. It was grown in medium Y supplemented with 0.1 mg/ml of adenine, 0.1 mg/ml of DL-methionine, 0.2 % yeast extract and 2 % glucose. The generation time was 2 hr at 30°. The cells were harvested after reaching the stationary phase and stored frozen at -20°.

Escherichia coli, strain K12 58-161, also known as W6, (Alföldi et al., 1962) which requires methionine and has a relaxed amino acid control of RNA synthesis, was grown and starved for methionine as previously described (Svensson et al., 1963).

Preparation of tRNA.

Methyl-deficient tRNA was prepared from methionine starved E. coli W6 and S. cerevisiae D38 as previously described (Svensson et al., 1963), except that the extraction with methyl cellosolve and the stripping was omitted.

Assay of enzyme activity.

The methylation of tRNA was measured as the amount of radioactive methyl groups transferred from (methyl-

^{14}C)SAM to tRNA. Methyl-deficient tRNA from methionine starved E. coli W6 was used as a general substrate for different methylases.

The reaction mixture for standard assay contained in a total volume of 0.2 ml: 0.1 M Tris-HCl, pH 8.0, 2 mM GSH, 0.1 mM EDTA (disodium), 10 mM $MgSO_4$, 20 mM NH_4Cl, 0.33 mμmoles of (methyl-^{14}C)SAM, 0.08 mg of E. coli tRNA and 0.1 ml of the enzyme fraction to be tested.

When tRNA from S. cerevisiae D38 became available, it was used as a specific substrate for tRNA (2DMG) methylase. The same reaction mixture was used except for 0.06 mg of yeast tRNA and 1.0 mμmoles of (methyl-^{14}C)SAM.

Incubation was for 60 min at 30°. Under these conditions, the apparent reaction rate is linear up to 90 min or more. The reaction was stopped with 2 ml of 0.01 M $La(NO_3)_3$ in 0.5 M $HClO_4$, and the precipitate was washed as described by Boman et al. (1961). The radioactivity was measured in a Nuclear Chicago windowless gas flow counter.

Characterization of the reaction product.

The standard assay, which measures incorporation of methyl groups into E. coli tRNA on the polynucleotide level, does not discriminate between the different tRNA methylases. The following procedure for product characterization was therefore worked out.

The reaction mixture for methylation of methyl-deficient tRNA from E. coli W6 contained in 1.0 ml: 0.1 M Tris-HCl, pH 8.0, 2 mM GSH, 0.1 mM EDTA, 10 mM $MgSO_4$, 20 mM NH_4Cl, 5.0 mμmoles of (methyl-^{14}C)SAM, 2.0 mg of tRNA and 0.5 ml of enzyme. After incubation for 12 hr at 30°, the reaction was stopped and the tRNA regained by two phenol extractions and two ethanol precipitations (Svensson et al., 1963). The precipitate was washed twice with 0.5 ml of 90 % ethanol (-15°), dried in a stream of air and dissolved in 60 μl of distilled water.

An aliquot of 25 μl was transferred to a small Pyrex glass tube (3mm x 30mm), and 25 μl of 2 M HCl was added. The solution was kept frozen while sealing the tube. The tRNA was hydrolyzed for 30 min at 100°, resulting in free

purine bases and pyrimidine nucleotides. An aliquot of 5 µl was subjected to two-dimensional thin-layer chromatography in solvents I and II on cellulose with methylated purines as markers. Details have been published previously (Björk and Svensson, 1967). This procedure resolves the 8 methylated adenines and guanines found *in vivo*. Since the methylated pyrimidine nucleotides do not migrate in solvent I, they appear together with other charged material along the edge of the chromatogram between the starting point and the front of solvent II. This edge was stripped, each strip being 1.5 cm wide and 0.5 cm in the direction of solvent II migration.

Perchloric acid hydrolysis.

The material to be hydrolyzed was dried and heated with 0.5 ml of 70 % $HClO_4$ to 100° for 2 hr. After dilution with 2 ml of distilled water, the solution was neutralized with 5M KOH. The precipitate was washed twice with cold water, and the combined solutions were taken to dryness. The hydrolyzed material was dissolved in a proper volume of water before application to a chromatography paper.

Phosphatase treatment.

Nucleotide material not exceeding 0.2 mg was dissolved in 0.5 ml of 0.1 M Tris-HCl, pH 8.9, containing 1 mM $MgCl_2$, and treated with 0.1 mg of alkaline phosphatase for 4 hr at 37°.

Paper chromatography.

Whatman No. 3MM paper was used except where otherwise stated. Chromatograms were run descending at 24°.

Solvent systems.

Solvent I: 1-butanol-H_2O (86:14, v/v), NH_3(gas).
Solvent II: 2-propanol-conc. HCl (170:41, v/v, water to 250).
Solvent III: 2-propanol-H_2O-conc. NH_3 (7:2:1, v/v).
Solvent IV: 2-propanol-H_2O-conc. NH_3 (85:15:1.3, v/v).

Molecular sieve chromatography.

Sephadex G-150, 40-120 μ, was obtained from AB Pharmacia, Uppsala, Sweden. The gel was allowed to swell for more than 24 hr in the buffer to be used and then deaerated. Packing and running the column was performed at 4°. Upward flow elution was used on account of the high concentration of the protein solution applied. The columns used were of a type designed at this institute (Porath and Bennich, 1962). Constant flow was achieved by means of a Mariotte flask or a peristaltic pump.

Hydroxylapatite chromatography.

Hydroxylapatite was prepared according to the procedure of Tiselius et al. (1957). Regeneration of the column was carried out with 1 M potassium phosphate buffer, pH 6.8, followed by several dead volumes of starting buffer. The equilibration was checked by measuring the conductivity of the effluent. The chromatograms were developed with potassium phosphate buffers as described in the experimental section.

Other methods.

Protein and tRNA concentrations were estimated from the absorbance at 280 mμ and 260 mμ, respectively, in a Zeiss spectrophotometer. A solution of 1 mg/ml of tRNA gives an absorbance of about $A_{260} = 25$ (1 cm light path).

RESULTS

Preparation of crude extract.

All procedures were performed at 0 - 5° unless otherwise stated. Enzyme fractions which were not immediately processed were stored at 0°, since freezing of more purified fractions has been found deleterious.

150 g of washed and frozen S. cerevisiae D84 cells were thawed and suspended in 15 ml of 0.02 M Tris-HCl, pH 7.6, containing 0.02 M $MgAc_2$. The suspension was made 5 mM in 2-ME and 1 mM in EDTA, and 1.5 mg of DNase was added. The cells were disintegrated by pressing (French

and Milner, 1955). The extract (150 ml) was diluted with 230 ml of 0.01 M Tris-HCl, pH 7.6, containing 5 mM 2-ME and 1 mM EDTA. The solution was made 0.01 M in $MgAc_2$ and centrifuged at 78,000g for 60 minutes in a Spinco preparative ultracentrifuge, rotor No. 30. For each tube, the clear, intermediary layer was carefully removed by means of a syringe. The turbid top layer and the cloudy bottom layer, including the pellet, was discarded. The removed solution was centrifuged at 78,000g for 150 minutes, causing the ribosomes to sediment. Again, the intermediary layer was removed by suction through a syringe. This solution is called crude extract and had a protein content of about 6 mg/ml and a ratio A_{280}/A_{260} mμ in the range of 0.6 - 0.7.

Ammonium sulfate precipitation I.

The crude extract (280 ml) was incubated at 30° for 60 min with 2 μg/ml of RNase. After dilution with 440 ml of 0.01 M Tris-HCl, pH 7.6, containing 5 mM 2-ME and 1 mM EDTA, solid ammonium sulfate was added to give 80 % saturation (404g) without adjustment of pH. After standing for 1 hr, the precipitate was collected by centrifugation at 20,000g for 60 min and dissolved in 200 ml of 0.05 M Tris-HCl, pH 7.6, containing 5 mM 2-ME and 1 mM EDTA. The solution was dialyzed overnight against 5 l of the same buffer. This solution, AS-I, had a protein content of about 6 mg/ml and a ratio A_{280}/A_{260} mμ of 0.8 - 0.9.

Ammonium sulfate precipitation II.

To the dialyzed AS-I (230 ml) was added solid ammonium sulfate to give 30 % saturation (41 g). The suspension was allowed to stand for 1 hr before centrifugation at 20,000g for 60 min. To the supernatant fluid was added 66 g of solid ammonium sulfate (70 % saturation), and the mixture was left for 1 hr. The precipitate was collected by centrifugation at 20,000g for 60 min and dissolved in 0.05 M Tris-HCl, pH 7.6, containing 5 mM 2-ME, 1 mM EDTA and 0.02 % sodium azide. The volume of this solution, AS-II, was chosen to give a protein concentration of 50-60 mg/ml.

The ratio $A_{280}/A_{260\ m\mu}$ was in the range of 1.2 - 1.4.

In some experiments, the removal of material insoluble in 30 % saturated ammonium sulfate was omitted. This change does not seem to alter the chromatographic patterns to follow.

Molecular sieve chromatography.

Fraction AS-II was immediately chromatographed on Sephadex G-150. As shown in Fig. 1A, the methylase activity, measured as incorporation of methyl groups into methyl-deficient tRNA from E. coli W6, was separated into two groups, peak I and peak II.

The individual methylating enzymes were assayed over the chromatogram by the thin-layer chromatography technique. Since the recovery of tRNA from the reaction mixture is to some extent irreproducible, a direct comparison between different samples cannot be made. For this reason, the following procedure was used. One portion (0.1 ml) of each enzyme sample was incubated for 60 min at 30° with methyl-deficient tRNA from E. coli W6 to determine the rate of methyl group incorporation with all the enzymes. Another portion (0.5 ml) of each enzyme sample was incubated similarly for analysis, by the thin-layer chromatography technique, the proportions between the different methylases. The values obtained were then applied to calculate the contribution of each individual methylase to the over-all rate of methylation. The result is shown in Fig. 1B.

As can be seen in the figure, the two main activities determined as tRNA (MU) methylase and tRNA (2DMG) methylase were well separated. The 2MG forming activity appeared together with the tRNA (2DMG) methylase activity and may partly reflect incomplete synthesis of 2DMG.

The activity denoted Y in Fig. 1B refers to all the radioactivity recovered from the thin-layer chromatogram in the region between the starting point and Cp (See Fig. 1 in Björk and Svensson, 1967), where depurinated, ribose-methylated dinucleotides are expected to appear (Isaksson and Phillips, 1968). It cannot at present be

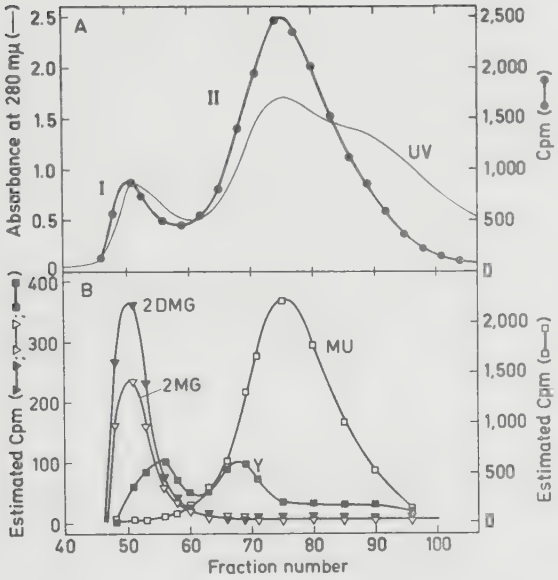

FIGURE 1

Molecular sieve chromatography of AS-II on Sephadex G-150. Column dimensions: 8 cm^2 x 90 cm. Eluting buffer was 0.05 M Tris-HCl, pH 7.6, containing 5 mM 2-ME, 1 mM EDTA and 0.02 % sodium azide. Flow rate was 17 ml/hr, and fraction volume was 5.5 ml. An aliquot of 8 ml of AS-II was applied.

A: (——), protein concentration expressed as A_{280}; (●—●), standard assay for methyl group incorporation into methyl-deficient tRNA from E. coli W6, expressed as uncorrected cpm.

B: Specific assay for different tRNA methylases using the thin-layer technique as described in the text. (▽—▽), tRNA (2MG) methylase; (▼—▼), tRNA (2DMG) methylase; (□—□), tRNA (MU) methylase; (■—■), tRNA methylase producing unidentified compounds (Y).

stated whether this activity represents the occurrence of one or more tRNA-ribose methylases.

In addition to the activities shown in Fig. 1B, the two peaks I and II contained other methylases, which did not give sufficient incorporation into tRNA to permit a reliable determination with the technique used. Traces of tRNA (1MG) methylase activity were spread out over the whole chromatogram. Table I shows that the enzyme was present in peak II. Its presence in peak I could also be demonstrated using prolonged incubation with pooled and concentrated protein.

tRNA (1MA) methylase was found in peak I only. Enzymes for 7MG and MC formation were not detected in the chromatogram, but showed up later in the fractionation procedure.

Chromatography of peak I on hydroxylapatite.

The fractions of peak I in Fig. 1 were pooled and adjusted with 1 M KH_2PO_4 to pH 6.8 and a final phosphate concentration of 0.02 M. The pooled material from two parallel Sephadex chromatograms was concentrated to half the volume by negative pressure dialysis and chromatographed on hydroxylapatite (Fig. 2). Two peaks of inactive material were eluted with the starting buffer and with 0.2 M potassium phosphate buffer.

TABLE I

Analysis of methyl-deficient tRNA from E. coli W6 methylated with different yeast enzyme fractions. Incubation was performed as described in Materials and Methods. In the case of peaks I and II, an additional amount of 0.25 ml of enzyme was added after 3 hr. The methylated constituents of tRNA were analyzed by the thin-layer chromatography technique. Compound Y summarizes all radioactivity found in the region between the starting point and Cp. Recovery is expressed in percent of total amount af radioactivity applied to the thin-layer plate. Counting of radioactivity was for 10 min. All values lower than 10 cpm above background have been omitted. 2MA, 6MA and 6DMA are not listed, since no activity has ever been found for these bases.

TABLE I

Enzyme source	Total amount recovered (cpm)	Recovery (%)	Recovered radioactivity (% of total ^{14}C recovered)							
			MUp	MCp	2DMG	2MG	7MG	1MG	1MA	Y
Peak I pooled (Fig. 1)	794	63	-	-	73	20	-	-	2	5
Peak G1 pooled (Fig. 2)	317	73	9	32	-	-	18	-	4	32
Peak G2 pooled (Fig. 2)	2,700	65	-	-	35	28	-	1	1	35
Peak II pooled (Fig. 1)	662	79	82	-	11	2	-	5	-	-
Peak C1 (Fig. 4, tube 112)	581	77	12	74	-	3	3	-	-	7
Peak C2 (Fig. 4, tube 137)	521	82	17	23	-	-	15	2	-	42
Peak C3 (Fig. 4, tube 150)	578	69	91	8	-	-	-	-	-	-
Peak U1 (Fig. 4, tube 180)	1,815	83	95	2	-	-	-	-	-	-
Peak U2 (Fig. 4, tube 214)	4,970	80	100	-	-	-	-	-	-	-
Peak U3 (Fig. 4, tube 239)	1,176	72	60	-	16	3	-	-	-	20

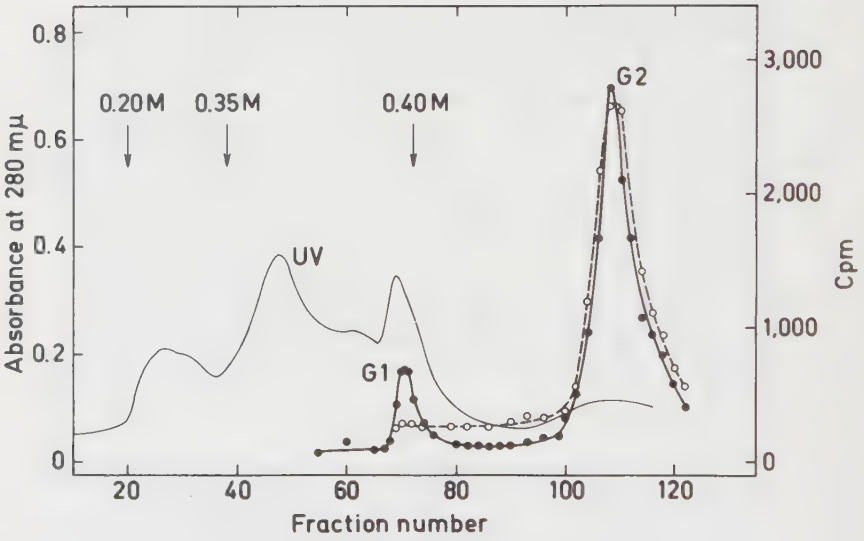

FIGURE 2

Hydroxylapatite chromatography of peak I from the molecular sieve chromatography on Sephadex G-150. Tubes number 47 - 56 in Fig. 1 were pooled and concentrated. A volume of 50 ml containing 0.1 g of protein was applied onto a column previously equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. Column size was 3 cm^2 x 40 cm. The flow rate was 12 ml/hr. Fraction time was 30 min in the beginning and 20 min from tube number 38 on. Stepwise elution of the protein was carried out with the following buffers: 0.01 M, 0.20 M, 0.35 M and 0.40 M potassium phosphate, pH 6.8. All buffers contained 5 mM 2-ME. Change of buffer is indicated by arrows. (——), protein concentration expressed as A_{280}. Standard assay for methyl group incorporation into methyl-deficient tRNA from *E. coli* W6 (●—●) and into *S. cerevisiae* D38 tRNA (○—○). All values are uncorrected cpm.

The methylase activity, assayed with methyl-deficient tRNA from E. coli W6, was then eluted in two well separated peaks, G1 with 0.35 M buffer and G2 with 0.40 M buffer. Both peaks were analyzed by thin-layer chromatography for different methylase activities, and the result is presented in Table I. Peak G1 contained enzymes for synthesis of MC and 7MG together with traces of activity for synthesis of 1MA and MU.

The product MCp was characterized by paper chromatography after phosphatase digestion to nucleosides. About 85 % of the radioactive material was recovered with almost the same R_{Cr}-value as reported for 5MCr (Table II). No radioactivity was found where 1MCr should appear. The radioactive material was eluted and further characterized by chromatography after hydrolysis to free bases with 5MC and 1MC as markers. All radioactivity appeared neatly under 5MC, well separated from 1MC (Table II).

Peak G2 contained all the enzymatic activity for synthesis of 2MG and 2DMG, and in addition traces of activity for 1MA and 1MG were found.

The exclusive location of tRNA (2DMG) methylase to peak G2 was also demonstrated using tRNA from S. cerevisiae D38 as specific substrate for this enzyme (Fig. 2).

Both peaks G1 and G2 had enzymatic activity for the formation of the product Y. The two products are not, however, identical, since they appear in different regions in the thin-layer chromatogram (Fig. 3). The R_{Cp}-values (in system II) of the products obtained with enzyme fractions G1 and G2 are 0.39 and 0.69, respectively. It is therefore possible that at least two different methylating enzymes are involved in the formation of product Y.

Chromatography of peak II on hydroxylapatite.

The pooled fractions of peak II in Fig. 1 were adjusted with 1 M KH_2PO_4 to pH 6.8 and a final phosphate concentration of 0.02 M. The pooled material from two parallel Sephadex chromatograms was concentrated about three-fold by negative pressure dialysis and chromatographed on hydroxylapatite (Fig. 4).

TABLE II

Chromatographic characterization of nucleosides and bases obtained after methylation of methyl-deficient tRNA from E. coli W6 with enzymes G1 and C1. The methylated tRNA was hydrolyzed to free purines and pyrimidine nucleotides and chromatographed in solvent II. The nucleotide material found in the MCp region (14,300 cpm for C1 and 1,490 cpm for G1) was degraded to nucleosides with alkaline phosphatase and chromatographed in solvent III. The radioactive material was eluted, hydrolyzed to free bases with $HClO_4$ and chromatographed in solvents III and IV on Whatman No. 1 paper. Literature data are from Hall (1965).

		Solvent		
		III R_{Cr}	III R_F	IV R_F
Nucleosides formed by enzyme:				
	G1	1.07	-	-
	C1	1.07	-	-
Literature data:	1MCr	1.40	-	-
	5MCr	1.00	-	-
Bases formed by enzyme:	G1	-	0.56	-
	C1	-	0.56	0.42
Markers:	5MC	-	0.56	0.42
	1MC	-	0.60	0.49

After elution of a large amount of inactive material with 0.10 M potassium phosphate buffer, the first methylating activity was eluted with 0.20 M buffer. Using a linear gradient, the resolution of the methylating activity into a total of six distinct peaks was achieved. The material in these peaks was analyzed for specific methylase content using the thin-layer chromatography technique. The results are presented in Table I.

With enzyme from peak C1, the main product formed was MCp. To characterize this compound further, it was degraded

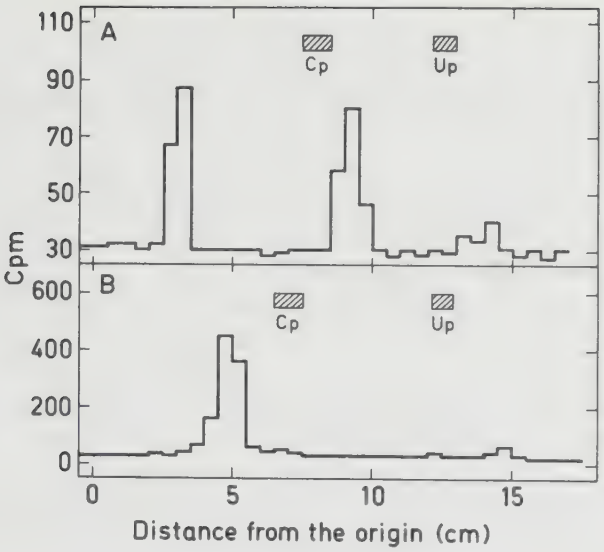

FIGURE 3.

Histogram of the nucleotide region on two-dimensional thin-layer chromatography, showing the different positions of compounds Y. The second peak to the right of Cp in upper part A is MCp. Methyl-deficient tRNA from E. coli W6 was methylated with enzyme fractions G1 (upper part A) and G2 (lower part B). The thin-layer plate was stripped as described under Materials and Methods.

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with alkaline phosphatase and chromatographed on paper with cytidine as marker. 77 % of the radioactivity was recovered with almost the same R_{Cr}-value as reported for 5MCr (Table II). No radioactivity was found where 1MCr should appear. The radioactive material was eluted, hydrolyzed to free bases and characterized by paper chromatography in two systems with 5MC and 1MC as markers. All radioactivity appeared with 5MC, well separated from 1MC (Table II).

The main product synthesized by enzyme from peak C2 was Y. Smaller amounts of MU and MC were also formed. The appearance of 7MG, which was not found in peak II of Fig.1, should be noted.

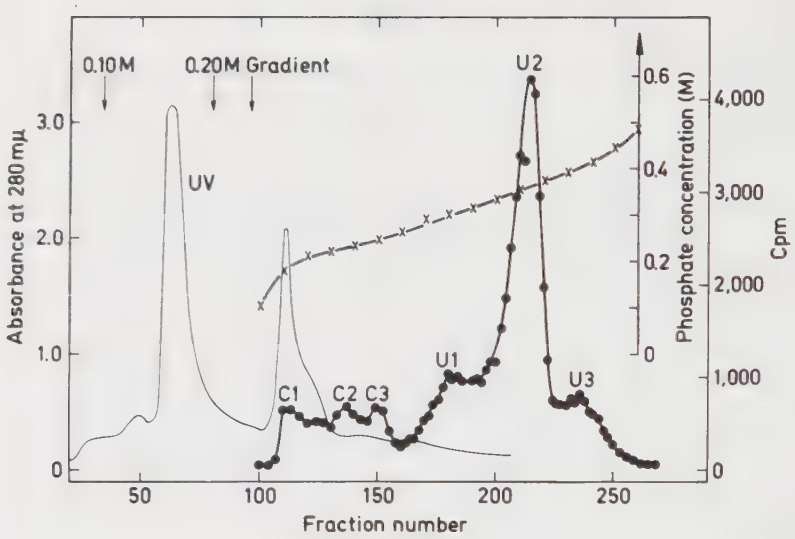

FIGURE 4.

Hydroxylapatite chromatography of peak II from the molecular sieve chromatography on Sephadex G-150. Tubes number 68 - 92 in Fig. 1 were pooled and concentrated. About 0.5 g of protein was applied onto a column previously equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. Column size was 3 cm^2 x 40 cm. The flow rate was 11 ml/hr. Fraction time was 20 min. Stepwise elution of the protein was carried out with 0.01 M, 0.10 M and 0.20 M potassium phosphate buffer, pH 6.8, followed by a linear gradient elution. The mixing chamber contained 300 ml of 0.20 M and the reservoir 300 ml of 0.50 M potassium phosphate buffer, pH 6.8. Changes of buffers are indicated by arrows. (——), protein concentration expressed as A_{280}. (●—●), standard assay for methyl group incorporation into methyl-deficient tRNA from E. coli W6. (x—x), phosphate concentration. All values are uncorrected cpm.

The remaining peaks C3, U1, U2 and U3 all contain enzyme activity for formation of MU as the dominating product. Usually there is not much activity for other pro-

ducts found in these peaks. Sometimes peak U3, however, may contain activities for 2DMG and Y, which was the case in the experiment shown in Table I.

The products obtained with enzymes in peaks U1, U2 and U3 from a chromatogram similar to that in Fig. 4 were characterized by paper chromatography. For each enzyme fraction, tRNA was methylated and subjected to acid hydrolysis resulting in purine bases and pyrimidine nucleotides. The hydrolyzates were chromatographed on paper in solvent II. The left part of Fig. 5 shows that with all three enzyme fractions the product is found exclusively in the MUp region.

The radioactive nucleotide material in each peak was eluted and divided into two parts. One half was subjected to perchloric acid hydrolysis, and the free bases formed were chromatographed on paper in solvent I. The result is shown in the right part of Fig. 5.

The other half of the radioactive nucleotide material was treated with alkaline phosphatase and chromatographed on paper as nucleosides. The R_F- and R_{Ur}-values obtained are given in Table III together with literature data for the authentic markers 1MUr and 5MUr.

With all the enzyme fractions U1, U2 and U3, the product formed is obviously the same. From the data of Fig. 5 and Table III we conclude that this product is 5MU.

The main product of enzyme fraction C3 has not been characterized.

The purification procedure for the tRNA (2DMG) methylase is summarized in Table IV and for one of the tRNA (5MU) methylases in Table V. The data given for total activity should be interpreted with caution, since the rate of reaction with unpurified fractions is enzyme-dependent in a non-linear fashion.

It should be pointed out that the data for total activity listed in Table V for all enzyme fractions except pooled peak U2 include other methylase activities besides the tRNA (5MU) methylase activity.

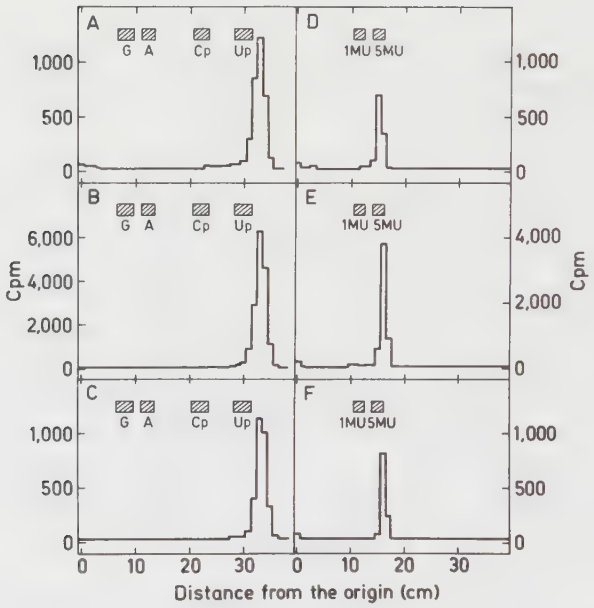

FIGURE 5.

Chromatographic characterization of products obtained after methylation of tRNA with enzymes U1, U2 and U3. Methyl-deficient tRNA (0.9 mg) from E. coli W6 was methylated and hydrolyzed as described for thin-layer chromatography analysis. The hydrolyzates were chromatographed on paper in solvent II for 24 hr at 25°.

A: Product formed by enzyme fraction U1.
B: " " " " " U2.
C: " " " " " U3.

Half of the radioactive material in each peak was hydrolyzed in $HClO_4$ and chromatographed on paper in solvent I for 15 hr at 25° with 1MU and 5MU as markers.

D: Hydrolyzate from A.
E: " " B.
F: " " C.

The chromatograms were cut into 1 cm strips, and the radioactivity was counted for 10 min in a liquid scintillation counter.

TABLE III

Chromatographic characterization of nucleosides obtained after methylation of methyl-deficient tRNA from E. coli W6 with enzymes U1, U2 and U3. The nucleotide material of the radioactive peaks in Fig. 5 A, B and C was degraded to nucleosides with alkaline phosphatase and chromatographed on paper in solvent III for 18 hr at 25°. Literature data are from Hall (1965).

		R_F	R_{Ur}
Product formed by enzyme:			
	U1	0.55	1.28
	U2	0.54	1.26
	U3	0.54	1.26
Marker:	Ur	0.43	-
Literature data:	Ur	0.38	-
	1MUr	0.70	1.84
	5MUr	0.54	1.42

TABLE IV

Summary of purification procedure of tRNA (2DMG) methylase. The enzyme was assayed with tRNA from S. cerevisiae D38.

Enzyme fraction	Total protein (mg)	Total activity (Cpm/min)	Specific activity (Cpm/min.mg)	Yield (%)
Crude extract	1,600	245,000	150	100
Ammonium sulfate fraction I	1,500	180,000	120	73
Ammonium sulfate fraction II	1,300	178,000	140	73
Pooled peak I from Sephadex G-150 column	80	115,000	1,400	47
Pooled peak II from Sephadex G-150 column	650	30,000	46	12
Pooled peak G2 from hydroxylapatite column	1.3	24,000	18,000	10
Peak G2 concentrated (7x)	1.3	16,000	12,000	6.5

TABLE V

Summary of purification procedure of tRNA (5MU) methylase. Enzyme activity was measured with methyl-deficient tRNA from *E. coli* W6.

Enzyme fraction	Total protein (mg)	Total activity (Cpm/min)
Crude extract	1,300	1,000,000
Ammonium sulfate fraction I	1,200	670,000
Ammonium sulfate fraction II	860	500,000
Pooled peak I from Sephadex G-150 column	38	24,000
Pooled peak II from Sephadex G-150 column	460	300,000
Pooled peak U2 from hydroxylapatite column	3	20,000

DISCUSSION

The purification procedure described here resolves the tRNA methylase activity into eight fairly distinct enzyme fractions. Product analysis after methylation of methyl-deficient tRNA from *E. coli* W6 shows that these enzyme fractions contain activities for the synthesis of 1MA, 1MG, 2MG, 2DMG, 7MG, 5MU and 5MC. The presence of these methylated bases in tRNA from *S. cerevisiae* D84 has been confirmed in a labelling experiment *in vivo* (Phillips and Kjellin-Stråby, 1967), which also indicated the presence of 6DMA and 1MH. No activity *in vitro* has, however, been found for 6DMA in our enzyme fractions. The thin-layer chromatography method, which has been used for product characterization, does not separate 1MH from 2DMG completely. For this reason, part of our activity referred to as 2DMG may possibly be due to tRNA (1MH) methylase. On the other hand, the formation of 1MH probably requires preformed hypoxanthine, which has not yet to our knowledge been found in *E. coli* tRNA.

We have not found any methylase activity for synthesis of 2MA and 6MA. This is consistent with the data of Phillips

and Kjellin-Stråby (1967), who did not find these bases in tRNA from S. cerevisiae D84, although there are literature reports on the presence of both 2MA and 6MA in yeast tRNA (Littlefield and Dunn, 1958; Hall, 1965). It cannot be settled at present whether this discrepancy reflects strain differences or merely inadequacies of the analytical methods.

The activities for synthesis of 2MG and 2DMG have not been separated. Since the appearance of 2MG may reflect incomplete methylation of guanine with the tRNA (2DMG) synthetase, we cannot at present conclude that there is a tRNA (2MG) methylase present in G2.

Product characterization showed that enzyme fractions U1, U2 and U3 contained activity for synthesis of 5MU only. The physical separation of these activities together with data presented in the accompanying paper indicates that there may be at least three different enzymes for methylation of uracil into 5MU.

No activity for synthesis of 1MU was present in enzyme fractions U1, U2 and U3. Such an activity would not be expected, since a recent report (Dunn and Flack, 1967) makes it unlikely that yeast tRNA contains 1MU. Also, we have confirmed by analysis of 5MUp, isolated by Dr. J.H. Phillips from yeast tRNA labelled in vivo (Phillips and Kjellin-Stråby, 1967), that there is no 1MU present in tRNA from S. cerevisiae D84.

The presence of 1MC in yeast tRNA has been reported (Hall, 1965). Analysis of MCp, isolated from in vivo labelled yeast tRNA, revealed small amounts of 1MC. We have not, however, found any activity for formation of this base in enzyme fractions G1 and C1. The possibility is left open that it might be found in some other fraction.

The methylated products Y have not been characterized. They appear in the region of the thin-layer chromatogram where acid resistent derivatives of ribose-methylated dinucleotides are expected. It is therefore possible that they represent ribose-methylating activities, although the radioactivity in these products may also be due to methylation of pyrimidines which are not hydrolyzed off the acid resistent dinucleotides under the conditions employed.

It should be pointed out that there is a correlation between the synthesis of 7MG and one of the products Y appearing in the thin-layer chromatogram near this base. In all cases when 7MG has been found this Y-component also appeared. We cannot at present state whether this correlation means that the two (or more) enzymes responsible for the synthesis of these products have been purified together or that the separation of the two components was inadequate.

The chromatograms obtained on Sephadex gel and hydroxylapatite are reproducible if due care is taken to perform the preceding preparative steps identically. Some effects resulting in deviations from the chromatography patterns shown in this paper are discussed in the accompanying paper.

ABSTRACT.

Fractionation of enzymes, which catalyze the transfer of methyl groups from S-adenosyl-L-methionine to transfer RNA, from extracts of Saccharomyces cerevisiae has yielded eight separate methylating fractions. The products of methylation were identified by thin-layer chromatography of acid hydrolyzed tRNA with known reference compounds. Enzyme activities for synthesis of four methylated guanines, 1-methyladenine, 5-methylcytosine and thymine have been demonstrated. Three of the enzyme fractions produced thymine, apparently free of other methylating activity. Some fractions also contained enzymatic activity for formation of a heterogeneous group of compounds resistent to acid hydrolysis, probably related to ribose-methylated dinucleotides.

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STUDIES ON MICROBIAL RNA

Properties of tRNA methylases from Saccharomyces cerevisiae

by

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INTRODUCTION

In the preceding paper a fractionation procedure for yeast tRNA methylases was described. The method yielded eight different enzyme fractions, which were characterized by analysis of the products formed. Three of these fractions, U1, U2 and U3, contained activity for synthesis of 5MU[1)] and were usually free from other methylase activities. The other enzyme fractions were heterogeneous with respect to the products formed.

We present here the properties of some of these enzymes. The availability of a tRNA from the yeast strain Saccharomyces cerevisiae D38, lacking the base 2DMG (Phillips and Kjellin-Stråby, 1967), has made possible the study of tRNA (2DMG) methylase without interference from other methylases. The tRNA (5MU) methylases have been studied with methyl-deficient tRNA from Escherichia coli W6 as substrate. Evidence is given that there may exist three or more different enzymes for synthesis of 5MU on the tRNA polymer.

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1) Abbreviations are the same as in the preceding paper.

MATERIALS AND METHODS

Aerobacter aerogenes was grown and harvested as described for *E. coli* (Svensson et al., 1963). Preparation of tRNA from different sources was as reported previously (Svensson, 1967 a,b).

The standard assay for methylation of tRNA was as described in the preceding paper with the following exceptions. *E. coli* tRNA was assayed with 0.65 mμmoles/ml of (methyl-^{14}C)SAM (50.5 mC/mmole), yeast tRNA with 2.5 mμmoles/ml of SAM, *A. aerogenes* tRNA (1.1 mg/ml) with 0.85 mμmoles/ml of SAM, and wheat germ tRNA (0.5 mg/ml) with 1.25 mμmoles/ml of SAM. Samples of 0.2 ml were taken out from the reaction mixture at different times.

Degradation of ^{14}C-methylated tRNA for oligonucleotide analysis was performed with Taka diastase T_1 RNase (Sankyo Co. Ltd., Ginza, Tokyo). The hydrolysis was carried out in 0.05 M Tris-HCl, pH 7.6, for 19 hr at 37° with 180 units of enzyme for each mg of tRNA. This amount is a reasonable excess of enzyme for complete hydrolysis of the susceptible internucleotide bonds.

Paper chromatography of the digestion products was run descending at 24° in solvent V: 1-propanol-H_2O-conc. NH_3 (55:35:10, v/v).

Dithiothreitol was bought from Calbiochem.

Other materials and methods used were described in the preceding paper.

RESULTS

Effect of pH on rate of reaction.

Optimal activity for the tRNA (5MU) methylase of peak U2 was found in the pH range 7.5 - 8.5 when assayed in Tris-HCl buffer with methyl-deficient tRNA. The tRNA (2DMG) methylase activity was measured in potassium phosphate and Tris-HCl buffers using enzyme AS-II and tRNA from *S. cerevisiae* D38. Optimal activity was observed between pH 7 and 8.

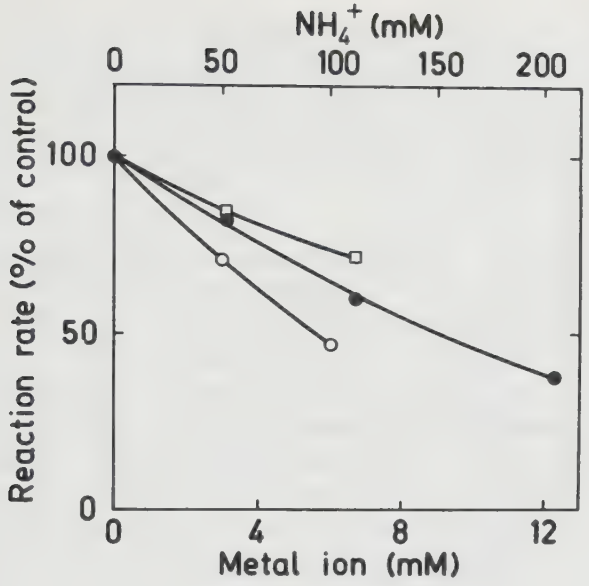

FIGURE 1

Effect of Mg^{2+} (●—●), Mn^{2+} (□—□) and NH_4^+ (○—○) on the rate of methylation with enzyme fraction U2. The reaction mixture contained in 1 ml: 0.1 M Tris-HCl, pH 7.4, 0.27 mg of methyl-deficient tRNA from E. coli W6, 1 mM GSH, 2.5 μmoles of (methyl-^{14}C)SAM, 0.5 mM EDTA, 0.7 ml of pooled enzyme fractions U2 and different additions of salts. The reaction rate in the control experiment was 766 cpm/20 min. A second control with 5 mM EDTA gave 709 cpm/20 min.

Effect of cations and sulfhydryl agents.

Fig. 1 shows that the tRNA (5MU) methylase of peak U2 is not activated by the divalent cations Mg^{2+} and Mn^{2+}. On the contrary, these ions inhibit the enzyme activity already at low concentrations. Also NH_4^+ has an inhibitory effect, lowering the reaction rate to about one half at 0.1 M. The tRNA (2DMG) methylase, on the other hand, is almost unaffected by Mg^{2+} in concentrations up to 0.01 M and only slightly inhibited by Mn^{2+} and Co^{2+} in the same concentrations. The enzyme activity was doubled by 0.05 M NH_4^+ (Fig. 2).

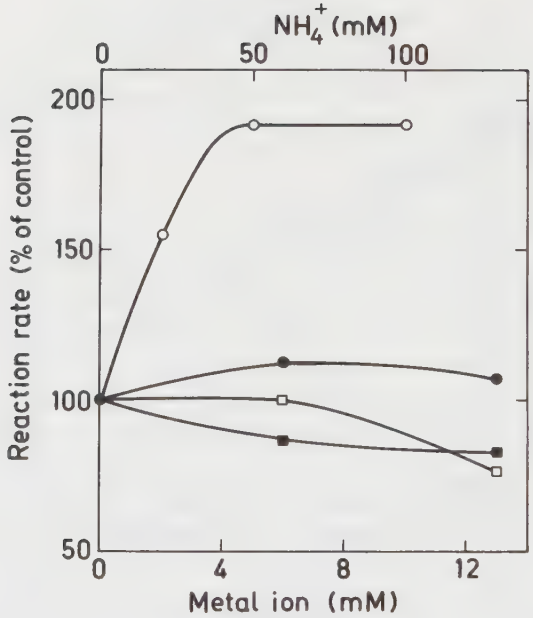

FIGURE 2

Effect of Mg^{2+} (●—●), Mn^{2+} (□—□), Co^{2+} (■—■) and NH_4^+ (○—○) on the rate of methylation with tRNA (2DMG) methylase. The reaction mixture for metal ion test contained in 1 ml: 0.1 M Tris-HCl, pH 7.6, 0.2 mg of tRNA from S. cerevisiae D38, 1 mM GSH, 1.8 mμmoles of (methyl-^{14}C)SAM, 1 mM EDTA, 0.7 ml of pooled enzyme G2, and different additions of salts. The reaction rate of the control, which contained 6 mM EDTA, was 295 cpm/30 min. When NH_4^+ was tested, the reaction mixture contained in 1 ml: 0.1 M Tris-HCl, pH 8.0, 0.3 mg of tRNA from S. cerevisiae D38, 5.0 mμmoles of (methyl-^{14}C)SAM, 0.1 ml of dialyzed fraction AS-I, and different additions of NH_4^+. The reaction rate in the control experiment was 239 cpm/30 min.

The effect of sulfhydryl agents on both enzymes was tested. The tRNA (5MU) methylase was greatly stimulated by the presence of 5 mM GSH or 2-ME in the reaction mixture, while the tRNA (2DMG) methylase was inhibited to some extent by GSH and apparently has no need for reduced sulfhydryl groups.

Enzyme dependence for reaction rate.

In the over-all methylation reaction using crude enzyme preparations and methyl-deficient E. coli tRNA, the rate of reaction was not proportional to the enzyme concentration. With increased enzyme concentration the specific activity decreased. The same result was obtained when tRNA (2DMG) methylase was specificly assayed with tRNA from S. cerevisiae D38. This behavior cannot be ascribed the presence of unlabelled SAM in the dialyzed crude extracts, since the effect was the same when an increased amount of ^{14}C-labelled SAM was used. With more purified enzyme fractions, proportionality between amount of enzyme and reaction rate was usually but not always found.

Requirement for SAM.

The Michaelis constant for SAM was determined with enzyme preparation AS-II and methyl-deficient E. coli tRNA. The value found, 0.6×10^{-5} M, thus represents all active tRNA methylases in the preparation. It is of the same order of magnitude as the Michaelis constants for some of the tRNA methylases from E. coli (Hurwitz et al., 1964).

Stability.

The stability of the different enzyme fractions varied from preparation to preparation. Most enzyme peaks lost activity rapidly when left at 0°. Enzyme G1, for instance, lost half of its activity in 12 days, and enzyme U2 lost 30 % of its activity in 5 days. The instability of tRNA (1MG) methylase is visualized by the rapid decrease in activity in the right peak of Fig. 4A. On the other hand, the tRNA (2DMG) methylase in peak G2 was stable at 0° for 14 days.

The stability of all enzymes tested was increased by storage in 30 % ethylene glycol at -20°, while freezing without antifreeze agents was deleterious.

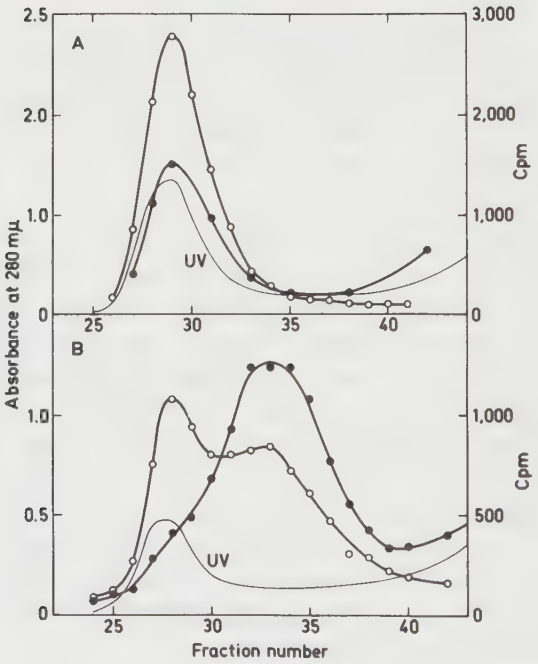

FIGURE 3

Molecular sieve chromatography on Sephadex G-150 of dialyzed (upper part) and undialyzed (lower part) enzyme fraction AS-I. Only the peak I region of the chromatogram is shown (cf. Fig. 1 in the preceding paper). Protein concentration is expressed as A_{280} (——). Enzyme activity, expressed as uncorrected cpm, was measured in standard assay using methyl-deficient tRNA from E. coli W6 (●——●) or tRNA from S. cerevisiae D38 (○——○). A: Enzyme AS-I was dialyzed for 17 hr against 0.05 M Tris-HCl, pH 7.6, containing 5 mM 2-ME and 1 mM EDTA. The column (2.8 cm^2 x 90 cm) was loaded with 80 mg of protein in a volume of 3 ml. Elution was with the same buffer, made 0.02 % in sodium azide. Flow rate was 9 ml/hr, fraction time was 20 min. B: Enzyme AS-I (130 mg protein in 6 ml) was applied without previous dialysis onto the column (8 cm^2 x 90 cm). Buffers were the same as in A. Flow rate was 18 ml/hr, fraction time was 30 min.

Dialysis of the methylases usually resulted in heavy loss of activity. The best pH to be used for dialysis of tRNA (2DMG) methylase and tRNA (5MU) methylase was found to be 8.5 and 6.5, respectively.

Effect of dialysis of enzyme fraction AS-I.

In the procedure for enzyme fractionation described in the preceding paper two active fractions, peaks I and II, were obtained after molecular sieve chromatography on Sephadex G-150 (Fig. 1 in the preceding paper). The procedure employed included dialysis of enzyme fraction AS-I. We observed that omission of the dialysis step usually led to different and less reproducible chromatograms on the gel. Peak I was broadened or split into two peaks as shown in Fig. 3, where a normal chromatogram (A) of dialyzed extract is compared with a chromatogram (B) obtained with undialyzed extract. It should be noticed that the figure only shows the region of peak I.

With dialyzed extract, the methylase activity of peak I was closely associated with the UV-absorbing material. When undialyzed extract was chromatographed, on the other hand, most of the activity was eluted in a second peak after the UV- absorbing material. The tRNA (2DMG) methylase, which was assayed independent of other methylases, appeared in both peaks. Since most probably there is only one methylase in yeast for synthesis of 2DMG, the data suggest that dialysis under our conditions favours the formation of a complexed tRNA (2DMG) methylase.

The two enzyme regions in Fig. 3 were analyzed for content of different methylating enzymes. Methyl-deficient tRNA from E. coli W6 was incubated for 5 hr, and the products were characterized by thin-layer chromatography after acid hydrolysis. The dominating product formed with enzyme from tube No. 28 was 2DMG (50 % of recovered radioactivity), and from tube No. 33 it was 1MG (40 % of recovered radioactivity). Almost no 1MG was found after methylation with enzyme from tube No. 28.

The profile of methylase activity after molecular sieving of undialyzed extract AS-I varied between different preparations. Part of this fluctuation can be explained by

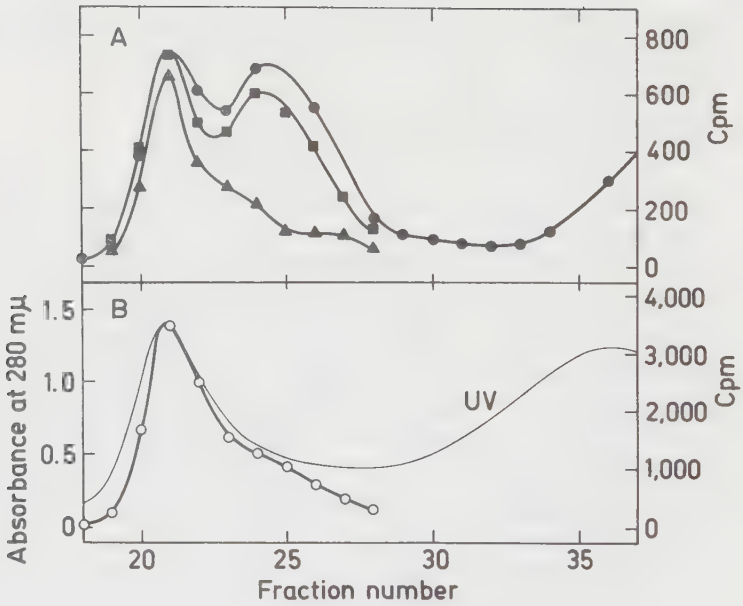

FIGURE 4.

Molecular sieve chromatography on Sephadex G-150 of undialyzed enzyme fraction AS-II. Only the peak I region of the chromatogram is shown (cf. Fig. 1 in the preceding paper). 270 mg of protein in 7 ml was applied on the column (8 cm^2 x 90 cm). Buffers used were the same as in Fig. 3. Flow rate was 17 ml/hr, fraction time was 40 min. Protein concentration is expressed as A_{280} (———). Enzyme activity, expressed as uncorrected cpm, was measured in standard assay using tRNA from S. cerevisiae D38 (○—○) or methyl-deficient tRNA from E. coli W6 (●—●). The assay with E. coli tRNA was repeated after 8 hr (■—■) and 93 hr (▲—▲). Product analysis after methylation has shown that activity for 1MG formation was present in tube No. 25 but not in tube No. 21.

TABLE I

Incorporation of ^{14}C-methyl groups into rRNA and tRNA with purified enzyme fractions. The reaction was done under standard assay conditions. Each sample analyzed contained 8 mg of methyl-deficient RNA from E. coli W6, with equal amounts of rRNA and tRNA.

Enzyme fraction	^{14}C-Methyl group incorporation into		
	rRNA (cpm)	tRNA (cpm)	rRNA (%)
G1	120	5,400	2.2
G2	150	19,800	0.8
C1	300	4,500	6.7
C2	190	6,400	3.0
C3	700	10,000	7.0
U1	800	15,600	5.1
U2	1,870	54,000	3.5

the different stability of the enzymes. In Fig. 4 it is shown that in a few days almost all the activity eluting after the UV- absorbing material of peak I had disappeared, while the enzymes associated with the UV-absorbing material had preserved almost full activity.

RNA and species specificity.

It was reported previously (Svensson et al., 1963) that yeast tRNA methylases did not significantly incorporate methyl groups into rRNA from E. coli W6, which had been starved for methionine. We have reinvestigated this problem, using the purified enzyme fractions eluted from the hydroxylapatite column. Total RNA, containing about equal amounts of tRNA and rRNA, was incubated with the different enzymes for 6 hr and regained by phenol extraction and ethanol precipitations. The distribution of radioactivity in tRNA and rRNA was measured after separation on Sephadex G-200 (Boman and Hjertén, 1962). Table I shows that all enzyme fractions tested incorporated methyl groups into rRNA in amounts varying between 1 and 7 % of the incorporation into tRNA. These

values should be interpreted with caution, because they probably do not represent saturation with methyl groups of the two RNA species. It should be kept in mind that the rRNA was methylated in a heterologous system and that the tRNA methylases may be more specific in a homologous system with submethylated yeast RNA. It is also possible that the activity towards rRNA is not an inherent property of the tRNA methylases but merely reflects the presence of rRNA methylases spread out over the chromatograms.

It has been shown (Srinivasan and Borek, 1964) that tRNA methylases from yeast, in accordance with methylases from other organisms, generally recognize new sites for methylation on a wide variety of heterologous tRNA's, which are already fully methylated with respect to their homologous enzymes. We have confirmed these results using a crude yeast enzyme preparation and tRNA from a number of different organisms. In some cases, the products obtained were identified by thin-layer chromatography of hydrolyzed tRNA. Table II shows that there is a great variation between organisms with respect to sites available for methylation with specific enzymes. Wheat germ tRNA seems to be a rather specific substrate for tRNA (5MU) methylase, while A. aerogenes tRNA is preferentially methylated to yield 2DMG and 1MG. There is no pronounced difference in the distribution of methylated bases between tRNA from logarithmically growing E. coli W6 and methionine-starved E. coli W6 cells after methylation with yeast enzyme. However, it must be pointed out that in crude extracts some methylases seem to be inactivated for reasons unknown.

The same tRNA preparations were also tested for methyl group acceptor ability with purified yeast methylase fractions. It can be seen from Table III that both methyl-deficient and normally methylated E. coli tRNA react with all enzyme fractions. The tRNA from A. aerogenes showed the best response with enzyme G2, which contained tRNA (2DMG) methylase, in agreement with the data in Table II. Wheat germ tRNA was active only with the enzyme fractions which contained tRNA (5MU) methylase as the dominating enzyme, also in accordance with Table II.

TABLE II

Product analysis of tRNA from different organisms after methylation with yeast enzymes. The enzyme was fraction AS-II, which was further purified by molecular sieve chromatography on Sephadex G-100. Standard assay conditions were employed. Incubation was for 5 hr. Compound Y summarizes all radioactivity found in the region between the starting point and Cp on the thin-layer plate. Recovery is expressed in percent of total amount of radioactivity applied to the thin-layer plate. All values lower than 10 cpm above background have been omitted.

tRNA source	Total amount recovered (cpm)	Recovery (%)	Recovered radioactivity (% of total ^{14}C recovered)							
			MUp	MCp	2DMG	2MG	7MG	1MG	1MA	Y
E. coli W6, starved for methionine	1,288	81	76	0	15	-	-	8	-	-
E. coli W6, log phase	427	72	70	-	21	7	-	-	-	-
S. cerevisiae D38	446	82	-	-	90	7	-	-	-	-
A. aerogenes	391	62	8	3	74	-	-	20	-	13
Wheat germs	1,098	82	98	-	-	-	-	2	-	-

TABLE III

Incorporation of ^{14}C-methyl groups into tRNA from different organisms with the purified enzyme fractions from hydroxylapatite chromatography. Standard assay conditions were employed. Reaction was for 1 hr in a volume of 0.2 ml. All values are given in cpm, corrected for blanks.

tRNA source	Enzyme source							
	G1	G2	C1	C2	C3	U1	U2	U3
E. coli W6, starved for methionine	790	1,430	270	460	740	1,380	4,590	560
E. coli W6, log phase	200	750	90	140	220	410	1,220	180
S. cerevisiae D38	0	940	-	-	-	-	-	-
A. aerogenes	140	1,170	100	90	10	40	190	140
Wheat germs	0	0	0	0	20	100	330	60

Specificity of the uracil methylating enzyme fractions.

The three enzyme fractions U1, U2 and U3, which were eluted successively from the hydroxylapatite column, all catalyze the formation of 5MU only (except that U3 sometimes also contained a few other activities). Although a buffer gradient was used for elution, the risk for chromatographic artifacts cannot be ignored, and the uracil methylating activity in the three peaks might therefore be due to the same enzyme. To examine this point, the following experiments were designed.

The maximum amount of methyl group incorporation into tRNA catalyzed by the three enzyme fractions was investigated. For each enzyme, the incorporation level was determined after incubation for 3 and 5 hr. In no case there was any further incorporation after the shorter reaction time, indicating that the plateau was reached in each case. Furthermore, the amount of methyl groups incorporated was directly proportional to the amount of tRNA present. It is evident from Fig. 5 that the three enzymes methylate E. coli

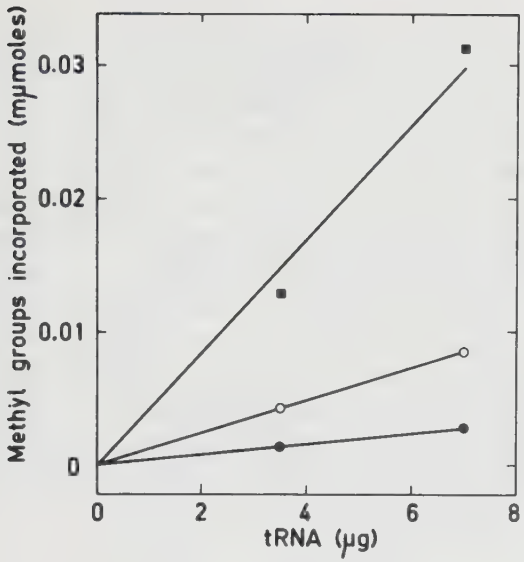

FIGURE 5

Extent of methylation as a function of the amount of tRNA. The reaction mixture contained in 1 ml: 0.1 M Tris-HCl, pH 7.4, 2 mM GSH, 0.1 mM EDTA, 10 mM Mg^{2+}, 20 mM NH_4^+, 4 mμmoles of (methyl-^{14}C)SAM, 0.8 ml of concentrated (5x) enzyme, and different amounts of methyl-deficient tRNA from E. coli W6. Incubation time was 3 and 5 hr for each value reported. Enzyme was from the left part of U1 (○—○), the middle part of peak U2 (■—■) and the right part of peak U3 (●—●).

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tRNA to different extents. This indicates that they differ in their specificity and thus are not identical. This conclusion is not affected by the fact that enzyme U3 used in this experiment contained some other methylase activities.

As a second approach to this problem, the three enzyme fractions were used for methylation of tRNA, which was then digested with ribonuclease T_1 and analyzed by paper chromatography. Fig. 6 shows that the digestion products from tRNA methylated with enzyme U2 differ from those obtained from tRNA methylated with enzymes U1 and U3. There is also a slight difference in distribution of the products with U1

and U3, but this may not be significant.

In some hydroxylapatite chromatograms we have observed a slight tendency for peak U2 to split into two parts. These observations suggested a heterogeneity in the methylase activity of this enzyme fraction. This has been verified by the experiment shown in Fig. 7. E. coli tRNA was first saturated with methyl groups, using enzyme from the front part of peak U2. It was ascertained that the saturation was complete by adding more of the same enzyme after reaching the plateau. No further incorporation was found. Under the same conditions addition of enzyme from the back part of peak U2, however, did increase the plateau level with 50 %. This shows that peak U2 contains at least two tRNA (5MU) methylases, which differ in their ability to recognize the sites for methylation on the tRNA polymer.

DISCUSSION

The properties of a single tRNA methylase can be meaningfully investigated only if the enzyme has been properly freed from other methylase activities or when it can be assayed specificly. Our purification procedure for the yeast tRNA methylases results in eight fractions, three of which contain methylase activity for the synthesis of 5MU exclusively. One of these fraction (U2) was chosen for many of the experiments reported, but it must be remembered that our results indicate the presence in fraction U2 of at least two different enzymes, which both catalyze the methylation of uracil to 5MU on the tRNA polymer.

For the investigation of tRNA (2DMG) methylase we ha had access to a highly specific tRNA from S. cerevisiae D38. When this tRNA is used as methyl group acceptor, there is no incorporation catalyzed by other methylases (Pillips and Kjellin-Stråby, 1967). The only methylated products formed are 2DMG and, as an intermediary precursor, 2MG. There is, for mutagenic reasons, most probably only one tRNA (2DMG) methylase in the yeast strain D84 used as enzyme source.

In our first paper on methylation of tRNA (Svensson et al., 1963) we reported an absolute dependence on Mg^{2+} for methylation with the yeast enzymes. However, since methionine

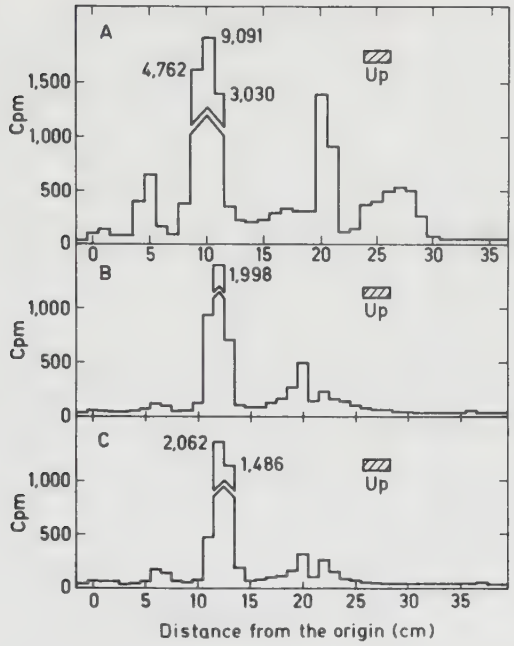

FIGURE 6

Paper chromatogram of ribonuclease T_1 digests of tRNA previously methylated by enzyme fractions U1, U2 and U3. The reaction mixture for methylation contained in 1.0 ml: 2.4 mg of methyl-deficient tRNA from E. coli W6, 0.55 ml of concentrated enzyme, 5.0 mμmoles of (methyl-^{14}C)SAM; other ingredients as in standard assay. Incubation was for 5 - 8 hr. The tRNA was regained, and an aliquot was degraded by ribonuclease T_1 and chromatographed for 22 hr at 24° in solvent V. A: 0.7 mg of tRNA methylated with enzyme U2. B: 1.4 mg of tRNA methylated with enzyme U1. C: 1.4 mg of tRNA methylated with enzyme U3. The scale of part A has been extended to allow direct comparison of peak positions relative to the marker Up between the chromatograms.

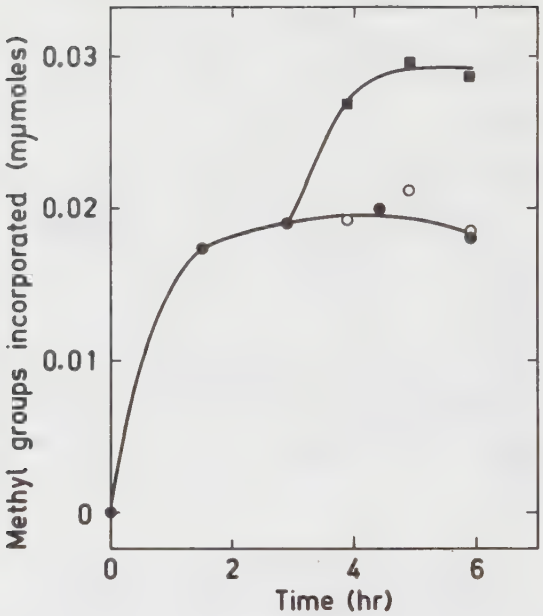

FIGURE 7

The extent of methylation of tRNA with enzyme from different parts of peak U2. The reaction mixture contained per ml: 0.02 mg of methyl-deficient tRNA from E. coli W6, 6.3 mμmoles of (methyl-^{14}C)SAM, 0.5 mM dithiothreitol, 0.6 ml of enzyme; other ingredients as in standard assay. (●—●), enzyme was from the front part of peak U2 (Cf. Fig. 4 in the preceding paper). Aliquots of 0.2 ml were taken out at different times and processed for counting. After incubation for about 3 hr, aliquots of 0.2 ml were also taken out for prolonged incubation with additions every 30 min of more enzyme (20 μl). One set was added with the same enzyme fraction (○—○), another set with enzyme from the back part of peak U2 (■—■).

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was used as the primary methyl donor, the need for Mg^{2+} might be due entirely to the enzymatic activation of methionine (Mudd and Cantoni, 1958). The results shown in Figs. 1 and 2 clearly demonstrate that the transfer of methyl groups from adenosylmethionine to tRNA with two of the yeast enzymes does

not require the presence of divalent metal ions. It has also been reported (Hurwitz et al., 1964 b) that the purified tRNA methylases from E. coli are active in the absence of divalent cations, although some of these enzymes are, in contrast to the yeast enzymes, effectively stimulated by Mg^{2+} and Mn^{2+}. Rat liver tRNA methylases have also been shown to function properly without Mg^{2+} (Rodeh et al., 1967).

The activity of the yeast tRNA (2DMG) methylase is stimulated 2-fold by NH_4^+. A similar effect was reported for the rat liver tRNA methylase preparation (Rodeh et al., 1967).

All our purified enzyme fractions are active not only with tRNA but also with rRNA from methionine-starved E. coli W6. We cannot at present determine whether the tRNA methylases are to some extent unspecific in the reaction with heterologous RNA or whether the enzyme fractions contain rRNA methylases. There are a few notes on the specificity of tRNA methylases towards rRNA. The rat liver enzyme preparation cannot methylate E. coli rRNA (Rodeh et al., 1967), and the purified tRNA methylases from E. coli have been reported to be inactive with methyl-deficient rRNA (Hurwitz et al., 1964 b), although in a later paper the same authors present data showing a small but possibly significant incorporation of methyl groups into rRNA (Hurwitz et al., 1965). Their paper also reports that the rRNA methylases from E. coli are active with tRNA as well.

It is doubtful if the ability of tRNA methylases to react with rRNA and of rRNA methylases to react with tRNA has a physiological meaning. If the enzymes themselves are somewhat unspecific, there must certainly exist means within the cell to prevent the expression of"crossed" reactions.

The species specificity of tRNA methylases from different organisms has been intensely studied (Srinivasan and Borek, 1963, 1964; Rodeh et al., 1967; Gold et al., 1963; Simon et al., 1967). All these studies show that tRNA methylases usually find new sites for methylation on heterologous tRNA's, which are already fully methylated by their homologous enzymes. Of special interest is the work on the purified tRNA methylases from E. coli (Gold et al., 1963), which demonstrates great variations between the individual enzymes in heterologous reactions. These findings are in accordance with

our results presented in Tables II and III.

Hydroxylapatite chromatography of methylating enzymes from yeast resulted in a fractionation of the tRNA (5MU) methylase activity into three distinct fractions, which could be obtained essentially free of other methylase activity. These fractions have been shown to possess different properties. They methylate E. coli to different extent (Fig. 5), they produce 5MU in different positions on tRNA as revealed by chromatography of ribonuclease T_1 digests (Fig. 6), and they methylate tRNA from A. aerogenes and wheat germs differently (Table III). Furthermore, one of the fractions has been shown to contain two activities which do not search out identical sites for methylation on the tRNA chains (Fig.7). All these results together with the physical separation on the column strongly indicate that yeast contains at least three or four different tRNA (5MU) methylases, which differ in their specificity. The existence of more than one methylase for the same product has also been demonstrated in E. coli through the separation of two tRNA (1MG) methylases (Hurwitz et al., 1964 a).

The presence of tRNA methylases synthesizing the same methylated base but differing in specificity is a support to the view that these enzymes may recognize specific nucleotide sequences on the tRNA polymer (Sarkar and Comb, 1966; Bagulcy and Staehelin, 1968). In this connection it is tempting to speculate over the possibility that different compartments in the cell may contain different sets of tRNA methylating enzymes. It has recently been shown that mitochondria in Neurospora crassa have their own tRNA chains and aminoacyl-tRNA synthetases, which differ in properties and specificity from those in the cytoplasm (Barnett et al., 1967; Barnett and Brown, 1967).

ABSTRACT

Partial purification of transfer RNA-methylating enzymes from Saccharomyces cerevisiae D84 yielded eight different enzyme fractions, the properties of which have been studied. All of these enzymes also showed some activity with

ribosomal RNA from Escherichia coli. The enzymes were found to differ in their species specificity. One of the enzyme fractions is highly active with Aerobacter aerogenes tRNA, forming N^2-dimethylguanine, but inactive with wheat germ tRNA. Other fractions are active with wheat germ tRNA, synthesizing 5-methyluracil only. Chromatography on hydroxylapatite resulted in three different fractions containing activity for the formation of 5-methyluracil in tRNA from E. coli. Although the same methylated base is formed, these enzymes differ in their specificities.

ACKNOWLEDGEMENTS

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STUDIES ON MICROBIAL RNA

Evidence for enzymatic methylation in vitro of the ribose moiety of transfer RNA

by

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INTRODUCTION

During the last few years the enzymatic methylation of nucleic acids has received increasing attention. It is now well established that the methylated bases are synthesized on the polynucleotide level with adenosylmethionine as methyl donor (Borek and Srinivasan, 1966; Srinivasan and Borek, 1966; Venkstern, 1967). The reaction is catalyzed by a set of specific enzymes, the properties of which have been studied in many laboratories.

Besides the methylated bases there is also a class of nucleosides in RNA with methyl groups in the 2'-O-position of the ribose moiety. Since Smith and Dunn (1959) first isolated 2'-O-methyladenosine from wheat germ and rat liver tRNA, five types of 2'-O-ribose methylated nucleosides have been found in tRNA from different sources (Hall, 1964). The distribution of the 2'-O-methylnucleosides in tRNA is non-random and differs from one species to another (Gray and Lane, 1967), which indicates some specificity in the biosynthesis of these compounds.

The mechanism of biosynthesis of the 2'-O-methylnucleosides has so far not been elucidated. However, several laboratories have some evidence for that *in vivo* the methyl group in the ribose part of tRNA originates from methionine (Dubin and Gänalp, 1967; Phillips and Kjellin-Stråby, 1967;

Nichols and Lane, 1967). There are at least two ways in which the methyl group could be built into the ribose moiety of tRNA. Either the ribose part of the mononucleotides becomes methylated prior to polymerization into tRNA or the methyl groups are incorporated on the polynucleotide level, i.e. after polymerization of the tRNA chain.

In this paper we describe a reaction *in vitro* with an enzyme preparation from *Saccharomyces cerevisiae* and tRNA from *Escherichia coli*, indicating that methylation of the ribose moiety in the 2'-O-position occurs on the polynucleotide level with SAM as methyl donor. The tRNA methylated *in vitro* was hydrolyzed in alkali and treated with phosphatase. The nucleosides and dinucleoside phosphates formed were separated by paper electrophoresis. The isolated dinucleoside phosphates were degraded enzymatically to nucleosides and analyzed by paper chromatography. One of the products formed has been identified as 2'-O-methyladenosine, and two others have been tentatively characterized as 2'-O-methylcytidine and 2'-O-methyluridine.

A preliminary account of this work has been published (Svensson et al., 1968).

Abbreviations: N, nucleoside; N_x, nucleoside containing a methyl group in the 2'-O-position of the ribose; N_xpN, alkali-stable dinucleoside monophosphate; N_xpNp, alkali-stable dinucleotide; dTpdT, thymidylyl-thymidine containing deoxyribose; other abbreviations as in the preceding papers.

MATERIALS AND METHODS

Chemicals.

Phosphodiesterase from *Vipera russelli* was purified through step 1 according to Björk (1963) and had $A^{1\ cm}_{280\ m\mu} = 11$. (Methyl-^{14}C)SAM (50.5 mC/mmole) was obtained from New England Nuclear Corp., Boston, Mass. 2'-O-methyladenosine and 2'-O-methylguanosine were generous gifts from Dr. R.K. Robins, University of Utah, Salt Lake City (Khwaja and Robins, 1966).

Enzyme preparation.

A crude extract containing RNA methylases was prepared from S. cerevisiae, strain D84, grown in rich medium, in the following way. 10 g of washed and frozen cells were thawed and suspended in 2 ml of ice-cold 0.02 M Tris-HCl, pH 7.7, containing 0.02 M $MgAc_2$, 5 mM 2-ME and 1 mM EDTA. 0.2 mg of DNase was added to the suspension, and the cells were disintegrated by pressing (French and Milner, 1955). After dilution with 15 ml of water, EDTA and 2-ME were added to give final concentrations of 1 mM and 5 mM, respectively. The extract was then incubated for 15 min at 30° and centrifuged for 60 min at 37,000g in the cold. To the supernatant fluid was added solid ammonium sulfate to give 80 % saturation, and the suspension was allowed to stand for 1 hr. The precipitate was collected by centrifugation for 20 min at 37,000g and dissolved in 15 ml of 0.01 M Tris-HCl, pH 7.7, which was 5 mM in 2-ME and 1 mM in EDTA. The solution was dialyzed against the same buffer for 20 hr and clarified by centrifugation for 60 min at 37,000g. A typical preparation had $A^{1\ cm}_{280\ m\mu}$ =26 and $A^{1\ cm}_{260\ m\mu}$ =34.

Methylation of tRNA in vitro.

The reaction mixture for methylation of tRNA contained per ml: 0.1 M Tris-HCl, pH 8.0, 2 mM GSH, 10 mM $MgCl_2$, 20 mM NH_4Cl, 0.1 mM EDTA, 2 mg of enzyme protein, 4 mg of tRNA from E. coli W6 grown in the presence of methionine, and 5 mμmoles of (methyl-^{14}C)SAM. After incubation for 7 hr at 30°, the tRNA was regained by two phenol extractions and two ethanol precipitations (Svensson et al., 1963). The precipitate was washed twice with 90 % ethanol.

Alkaline hydrolysis of tRNA.

Alkaline hydrolysis was performed in 0.2 M NaOH for 16 hr at 37° with 8 mg/ml of tRNA. The solution was then neutralized with Amberlite IRC-50 (H^+), which had been previously washed several times with 1 M NaOH, 1 M HCl and water. The neutralized solution was collected, and the resin was washed three times with water, previously adjusted to pH 8 with ammonia. The combined solutions were dried in a stream of air at 37°.

Dephosphorylation of nucleotides.

The nucleotides and dinucleotides to be dephosphorylated were dissolved (15 mg/ml) in 0.1 M triethylammonium bicarbonate, pH 8.9, containing 1 mM $MgCl_2$, and incubated with 0.3 mg/ml of alkaline phosphatase for 6 hr at 37°. This treatment results in complete hydrolysis of monoesterified phosphates.

Digestion of dinucleoside monophosphates.

Dinucleoside monophosphates isolated from 15 mg of tRNA were dissolved in 1 ml of 0.1 M triethylammonium bicarbonate, pH 8.9, containing 1 mM $MgCl_2$, and incubated with 0.2 mg of alkaline phosphatase and 0.4 A_{280}-units of snake-venom phosphodiesterase for 21 hr at 37°. This treatment is sufficient for complete hydrolysis to nucleosides.

Periodate test.

The nucleosides to be tested for susceptibility to periodate oxidation were dissolved in 0.1 ml of water, and 0.05 ml of 0.1 M $NaIO_4$ was added. After standing for 30 min at 20°, the solution was chromatographed together with an untreated control in solvents I and VII for 15 hr. Those nucleosides that after periodate treatment showed the same chromatographic behavior as the control have been classified as periodate resistent.

Deamination of nucleosides.

The material to be deaminated was dried in a polyethylene tube and treated with 25 µl of 2 M $NaNO_2$ and 20 µl of glacial HAc for 1 hr at 20°. After new additions of the reagents, the treatment was for another hour. The solution was then directly applied onto paper for chromatography.

Paper chromatography.

Whatman No. 1 paper was used except where otherwise stated. Chromatograms were run descending at 24°. The paper was cut into 1 cm strips, and the radioactivity was counted for 10 min in a Nuclear Chicago Mark I liquid scintillation counter.

Solvent systems.

Solvent I: 1-butanol-H_2O (86:14, v/v), NH_3(gas).

Solvent VI: 1-butanol-H_2O-conc. NH_3 (86:14:5, v/v).

Solvent VII: ethyl acetate-1-propanol-H_2O (4:1:2, v/v); only the top phase was used.

Solvent VIII: 2-propanol-conc. HCl-H_2O (680:170:144, v/v).

Solvents II-V are the systems described in the preceding papers.

Other materials and methods used have been described in the preceding papers.

RESULTS

The presence of a methyl group in the 2'-O-position of a nucleoside confers alkali-stability on the internucleoside phosphodiester bond to the 3'-position of the nucleoside carrying the methyl group in its ribose moiety (Smith and Dunn, 1959). As a consequence, alkaline hydrolysis of a polyribonucleotide containing 2'-O-methylribose results in the formation of stable dinucleotides of the type N_xpNp (Singh and Lane, 1964) and possibly also trinucleotides of the type N_xpN_xpNp (Gray and Lane, 1967). This facilitates the purification of the ribose-methylated nucleosides.

Isolation of dinucleoside monophosphates.

A total amount of 0.1 g of tRNA from *E. coli* W6, grown in the presence of methionine, was methylated *in vitro* with enzymes from *S. cerevisiae* D84. The tRNA was recovered from the reaction mixture and hydrolyzed in 0.2 M NaOH for 16 hr at 37°, resulting in dinucleotides of the type N_xpNp in addition to 3'-nucleotides, nucleosides and nucleoside diphosphates. The alkaline hydrolysate was neutralized, dried and treated with alkaline phosphatase. The solution, which then contained nucleosides and dinucleoside monophosphates of the type N_xpN, was subjected to chromatography on Whatman No. 3MM paper in solvent I (1-butanol system) for 25 hr. The paper was loaded with about 1 mg of hydrolyzed material per cm. The nucleotide region (from 0.5 cm behind and up to 2.0 cm in front of the starting line), indicated by the position of the marker dTpdT, was eluted and subjected to paper electrophoresis at pH 7.2. A typical electrophoretogram of enriched dinucleoside

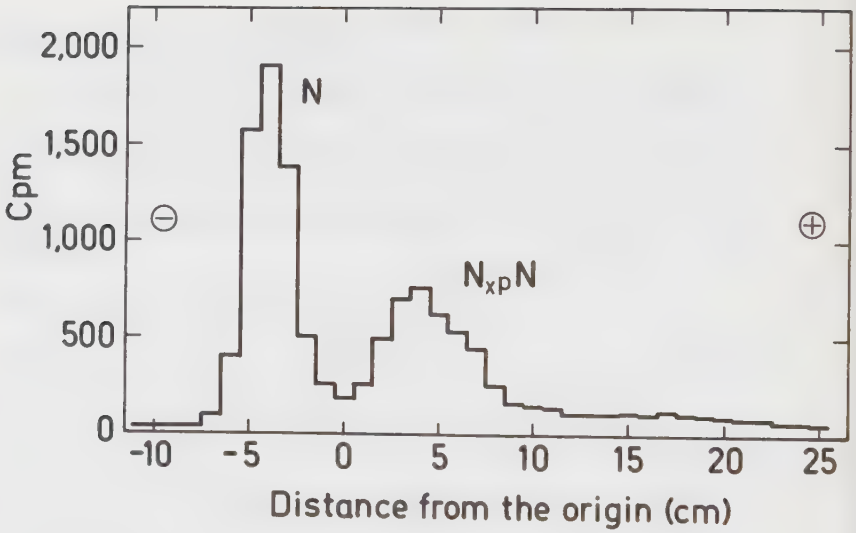

FIGURE 1.

Electrophoretogram of nucleosides (N) and dinucleoside monophosphates ($N_{x}pN$) recovered from chromatography in solvent I. The electrophoresis was carried out in 0.01 M potassium phosphate buffer, pH 7.2, (40 V/cm; 90 min), as described by Markham (1965). Whatman No. 3MM paper was used.

monophosphates is shown in Fig. 1. At the pH used the nucleosides slowly migrate towards the cathode, while the dinucleoside monophosphates migrate towards the anode.

Isolation of 2'-O-methylnucleosides.

The material in the dinucleoside monophosphate region of the electrophoretogram was eluted with 0.2 M ammonia and digested with snake-venom phosphodiesterase and alkaline phosphatase. The resulting nucleosides were chromatographed on paper in the ethyl acetate system. As shown in Fig. 2, the radioactive material was resolved into four peaks.

Each of these peaks was separately eluted and chromatographed in the 2-propanol system. Peak I was not further resolved, but gave rise to a very broad peak (Ia). The material in peak II was fractionated into four peaks (Table I). The fractionation of peaks III and IV is shown in Fig. 3 and Fig. 4, respectively.

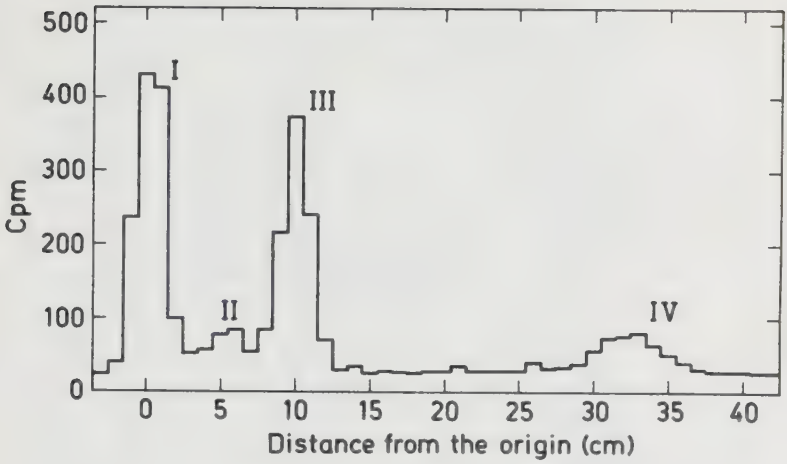

FIGURE 2.

Paper chromatography of products obtained after digestion of purified N_xpN with snake-venom phosphodiesterase and E. coli alkaline phosphatase. The chromatogram was developed for 15 hr in solvent VII (ethyl acetate system).

With a few exceptions, the resulting peaks were eluted and chromatographed in the 1-butanol system. The results from all these chromatograms are summarized in Table I. The fractionation of peaks IIId, IVb and IVd is shown in Figs. 3 and 4.

Characterization of the 2'-O-methylated nucleosides.

Ordinary ribonucleosides are sensitive to periodate oxidation, which breaks the bond between 2'-C and 3'-C in the ribose with the subsequent formation of a dialdehyde. The presence of a methyl group in the 2'-O-position renders the nucleoside resistent to periodate oxidation. Therefore, in order to select the ribose-methylated nucleosides from the multitude of products obtained by paper chromatography fractionation, the material in the major peaks was tested with periodate and chromatographed in two systems together with an untreated control. Those compounds, which had not changed their chromatographic behavior, were classified in Table I as resistent to periodate oxidation. The 2'-O-methylated nucleosides should belong to this class.

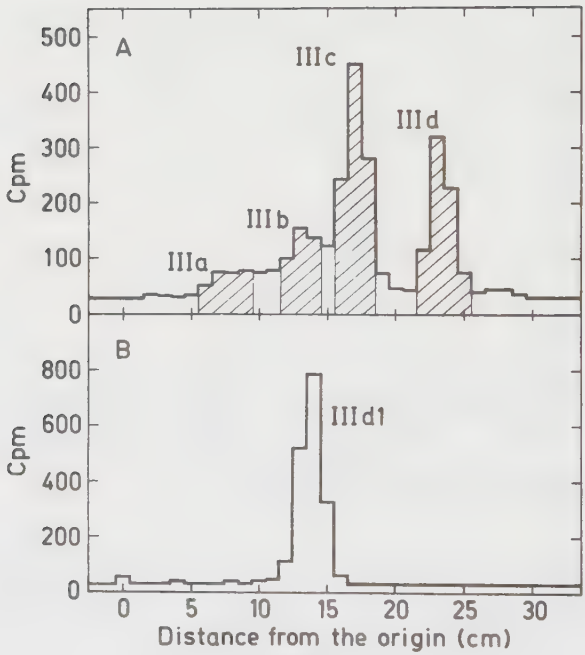

FIGURE 3.

Chromatographic fractionation of radioactive material recovered from peak III in Fig. 2. A: Paper chromatography in solvent VIII (2-propanol system) of the material from peak III. The chromatogram was run for 28 hr. The material in the hatched areas was eluted for further fractionation. B: Paper chromatography in solvent VI (1-butanol system) of the material recovered from peak IIId in part A. The chromatogram was run for 15 hr.

The periodate resistent compounds were chromatographed in solvent I with authentic 2'-O-methyladenosine and 2'-O-methyl-guanosine as references. Other authentic 2'-O-methyl-nucleosides were not available to us. The substance of peak IVb2 was found to migrate with the same rate as 2'-O-methyl-adenosine. It was therefore further characterized by chromatography in solvents VII and VIII. Table II shows that in all three systems this compound had the same R_{Ar}-values as 2'-O-methyladenosine.

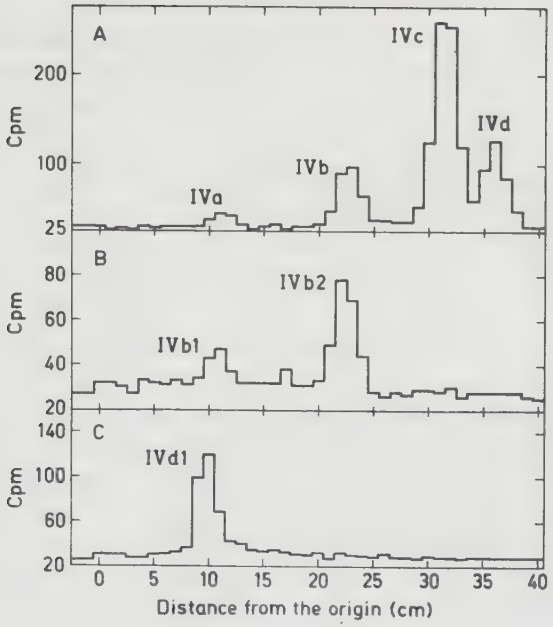

FIGURE 4.

Chromatographic fractionation of radioactive material recovered from peak IV in Fig. 2. A: Paper chromatography in solvent VII (2-propanol system) of the material from peak IV. The chromatogram was run for 28 hr. B: Paper chromatography in solvent VI (1-butanol system) for 15 hr of the material recovered from peak IVb in part A. C: Paper chromatography in solvent VI for 15 hr of the material recovered from peak IVd.

None of the compounds resistent to periodate oxidation had the same chromatographic properties as 2'-O-methylguanosine.

Hall (1965) has reported that 2'-O-methyladenosine and 2'-O-methyluridine have almost identical R_F-values in solvent VII, much different from those of other 2'-O-methylribonucleosides. According to his data, 2'-O-methyluridine should appear in peak IV and 2'-O-methylcytosine in peak III (Fig. 2). Furthermore, the R_{Ar}-values in solvents VI and VIII for two of

our products, IIId1 and IVd1, were in fair agreement with the data of Hall (1963). These compounds were therefore subjected to a deamination test using sodium nitrite, which transforms cytosine into uracil. If our assumption were right, compound IIId1 should give rise to a new product with the same properties as compound IVd1. Table III shows that this prediction came true.

Nitrite treatment of compound IIId1 and subsequent chromatography in solvent VIII resulted in two peaks, P1 and P2. Peak P2 contained 20 % of the radioactivity and had the same R_{Ar}-value as compound IVd1 in three different chromatography systems. Peak P1 had the same R_{Ar}-value in solvent VIII as the original peak IIId1 and consisted evidently of unreacted material, since renewed treatment with nitrite resulted in the formation of an additional amount (20 %) of peak P2 material. Treatment of compound IVd1 with nitrite did not change its R_{Ar}-value in solvent VIII.

The results obtained indicate that the material in peak IIId1 is 2'-O-methylcytidine and the material in peak IVd1 2'-O-methyluridine.

Characterization of the reaction.

It has been found that yeast enzymes may incorporate small but significant amounts of methyl groups into yeast tRNA (Srinivasan and Borek, 1964). Since our yeast enzyme preparation contained about 1.4 mg/ml of RNA, the possibility existed that the formation of the 2'-O-methylated ribonucleosides isolated was due to yeast RNA. To test this,

TABLE I

Chromatographic fractionation of nucleosides obtained by enzymatic degradation of electrophoretically purified alkali-stable dinucleoside monophosphates. The hydrolyzate was first chromatographed in solvent VII. The resulting peaks (I - IV) were further fractionated in solvent VIII. With a few exceptions the resulting peaks were chromatographed in solvent VI. The different fractions are characterized by their R_{Ar}-values. Recovery is given in cpm, corrected for background radiation. Compounds resistent to periodate oxidation are denoted with +, those which are sensitive with -.

TABLE I

Solvent VII			Solvent VIII			Solvent VI			Resistance to periodate oxidation	Product
Peak	R_{Ar}	Cpm	Peak	R_{Ar}	Cpm	Peak	R_{Ar}	Cpm		
I	0.00	6,410	Ia	1.86	6,400	Ia1	0.00	4,120		
						Ia2	0.87	400		
						Ia3	1.48	400		
II	0.36	850	IIa	0.77	60	IIc1	0.08	70		
			IIb	1.11	70	IIc2	0.42	60		
			IIc	1.39	260	IIc3	0.93	80		
			IId	2.21	300	IId1	1.48	260	-	
III	0.68	9,710	IIIa	0.65	470	IIIa1	0.00	30		
						IIIa2	0.39	50		
						IIIa3	0.82	360	+	
			IIIb	1.14	870	IIIb1	0.00	40		
						IIIb2	0.37	170		
						IIIb3	0.82	450	+	
			IIIc	1.45	2,700	IIIc1	0.08	380	-	
						IIIc2	0.46	1,040	-	
						IIIc3	1.01	530	+	
						IIIc4	1.60	130		
			IIId	1.98	2,510	IIId1	1.22	1,620	+	2'-O-MCr
IV	2.10	1,290	IVa	0.77	35	IVb1	0.94	40		
			IVb	1.59	170	IVb2	1.88	120	+	2'-O-MAr
			IVc	2.22	670	IVc1	0.43	670	-	
			IVd	2.54	220	IVd1	0.86	200	+	2'-O-MUr

TABLE II

Chromatographic comparison of product isolated from peak IVb2 with authentic reference compounds.

Compound	Solvent I R_{Ar}	Solvent VII R_{Ar}	Solvent VIII R_{Ar}
2'-O-methylguanosine	0.46	0.73	1.50
2'-O-methyladenosine	1.74	2.06	1.57
Peak IVb2	1.74	2.10	1.60

TABLE III

Chromatographic comparison of products from peaks IIId1 and IVd1 and of their deamination products.

Compound	Solvent VIII R_{Ar}	Solvent VII R_{Ar}	Solvent I R_{Ar}
Peak IIId1	2.10		
Peak IIId1 after nitrite treatment: P1	2.10		
P2	2.80	2.50	0.68
Peak IVd1	2.80	2.50	0.68
Peak IVd1 after nitrite treatment	2.80		

we made a control experiment, omitting the E. coli tRNA from the reaction mixture. Degradation and analysis of products was done as outlined above. No radioactivity was found in the regions where peaks III and IV (Fig. 2) should appear (Table IV). This proves that the 2'-O-methylnucleosides were synthesized on the E. coli tRNA polymer.

We have also excluded the possibility that the reaction leading to formation of 2'-O-methylnucleosides might be non-enzymatic. A control experiment was performed with the omission of enzyme from the reaction mixture. Table IV shows that there

TABLE IV

Recovery of radioactivity in methylated tRNA and in fractionated hydrolyzation products. All values are given in cpm, corrected for background radiation.

Incorporation into	Reaction mixture		
	Complete	Enzyme omitted	tRNA omitted
tRNA (0.1 g)	250,000	860	-
Nucleosides after electrophoresis	36,500	180	750
N_xpN after electrophoresis	18,260	80	150
Peak III	9,710	0	0
Peak IIId1 (2'-O-MCr)	1,620	-	-
Peak IV	1,290	0	0
Peak IVb	170	-	-
Peak IVb2 (2'-O-MAr)	120	-	-
Peak IVd1 (2'-O-MUr)	200	-	-

was no radioactivity to be found in the regions where peaks III and IV should appear. Thus the synthesis of the 2'-O-methylnucleosides requires the presence of enzymes.

DISCUSSION

Our results suggest that, in analogy with the biosynthesis of methylated bases in RNA, the 2'-O-methylated nucleosides are enzymatically synthesized on the polynucleotide level with S-adenosylmethionine as a possible methyl donor. One of the methylated products formed by the yeast enzyme in the presence of E. coli tRNA has been identified as 2'-O-methyladenosine. Two others were tentatively characterized as 2'-O-methyluridine and 2'-O-methylcytidine, but conclusive identification of these products has to await the availability of authentic reference compounds.

The remaining periodate resistent nucleosides have not been identified. Chromatography with authentic marker has shown, that none of them can be 2'-O-methylguanosine. Chromatographic data from the literature suggest that one of these compounds may be 2'-O-methylpseudouridine, but we have no other indications so far.

The yield of 2'-O-methylnucleosides isolated after reaction was very low. The reason for this is obscure, but some explanations may be suggested. One possibility is that E. coli tRNA present in the reaction mixture does not offer many sites to the yeast enzyme. It is also possible that the enzyme is rapidly inactivated during the reaction time before the sites available to ribose mathylation are saturated, or that the activity of the enzyme is not expressed due to inhibition or inadequate reaction conditions. Another explanation may be that our fractionation procedure has resulted in loss of ribose-methylated products.

S-adenosylmethionine was used as the only methyl donor. This compound seems to be the only methyl donor acceptable to the enzymes which methylate the bases in nucleic acids (Hurwitz et al., 1964 b). It is, however, possible that the enzymes responsible for ribose methylation require some other methyl donor.

It has been demonstrated that the tRNA-base methylases catalyze the formation of the same bases irrespective of the source of tRNA used (Hurwitz et al., 1964 a; Svensson et al., 1968 b). It would be surprising if the yeast enzymes which in our heterologous reaction in vitro produced the 2'-O-methylnucleosides should not give the same products in vivo.

Evidence is accumulating that the different methylated bases in tRNA are synthesized by a set of highly specific enzymes, at least one enzyme for each methylated base (Hurwitz et al., 1964 a; Björk and Svensson, 1968). It is probable that the synthesis of the ribose-methylated ribonucleosides is also mediated by a set of specific enzymes. The fractionation of the activity that produced compound Y (Björk and Svensson, 1968) into two peaks may be an indication that this is the case in yeast.

The result presented in this paper and in a preliminary report (Svensson et al., 1968 a) is the first evidence for enzymatic methylation *in vitro* of the ribose moiety in tRNA. In a recent paper, Nichols and Lane (1968) have demonstrated a 2'-O-methylation *in vitro* of rRNA, using ribosome precursor particles from methionine-starved *E. coli* W6 (RC^{rel}) and S-adenosylmethionine as methyl donor. Their data, together with ours, suggest that the mechanism for biosynthesis of the 2'-O-methylnucleosides is the same for tRNA and rRNA, in analogy with the biosynthesis of methylated bases in RNA.

It should be mentioned that a simple and rapid procedure for assaying ribose-methylating enzymes has recently been published (Baskin and Dekker, 1967). This method may be of value in estimating total ribose methylation, although it does not discriminate between the different 2'-O-methyl-nucleosides produced.

ABSTRACT

Methylation of transfer RNA from *Escherichia coli* in the 2'-O-position of the ribose has been achieved *in vitro* with an enzyme preparation from *Saccharomyces cerevisiae* and S-adenosylmethionine as methyl donor. The reaction occurs on the polynucleotide level. One of the products obtained is 2'-O-methyladenosine, two others have been tentatively identified as 2'-O-methylcytidine and 2'-O-methyluridine.

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